Submerged Fermentation of *Colletotrichum truncatum* for Biological Control of Scentless Chamomile

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ABSTRACT

An isolate of *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore is a promising bioherbicide candidate against scentless chamomile (*Matricaria perforata* Mérat), a noxious weed in western Canada. A major constraint in the development of this bioherbicide is the inefficiency of inoculum production. The objective of this study was to explore submerged fermentation for mass production of *C. truncatum*.

A defined basal salts medium (DBSM) was used for liquid culture with glucose and casamino acids selected as the optimal carbon (C) and nitrogen (N) sources, respectively. Spore yield and biomass production were significantly higher when the DBSM glucose concentration was 35-40 g/L compared to lower concentrations, while inoculum efficacy was significantly greater when produced at 5-10 g/L than at 40 g/L of glucose. Spore yield in baffled flasks at 200 RPM shaker speed was significantly higher than in regular flasks at lower shaker speeds. Under conditions of high aeration, glucose concentration had a significant effect on spore yield, biomass production, and efficacy, whereas the effect was not significant at low aeration. Specific spore and biomass yields also increased significantly with increasing glucose concentrations at high aeration. The scale of submerged fermentation was increased to 20-L fermentors, with dO levels of 10%, 30%, and 60% maintained by agitation and airflow controls. Further study will be required to optimize spore yields at the large scale.

This study led to development of a protocol for production of *C. truncatum* spores using submerged fermentation. Inoculum produced with this method can be used for laboratory, greenhouse, and field trials in development of the bioherbicide.

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
ANOVA	Analysis of variance
C	Carbon
CFW	Control fresh weight
CRD	Completely randomized design
DBSM	Defined basal salts medium
dO	Dissolved oxygen
DOT	Dissolved oxygen tension
FWR	Fresh weight reduction
IPM	Integrated pest management
Kla	Gas-liquid mass transfer
LSD	Least significant difference
N	Nitrogen
NA	Nutrient agar
PDA	Potato dextrose agar
ppm	Parts per million
PROC GLM	General linear model procedure
PSI	Pounds per square inch
RPM	Revolutions per minute
sp	Spores
SBY	Specific biomass yield
SSY	Specific spore yield
SRC	Saskatchewan Research Council
TFW	Treated fresh weight
V8	V8 juice
vvm	Volume of air per fermentor volume per minute

1. INTRODUCTION

Scentless chamomile (*Matricaria perforata* Mérat) is a Class-3 secondary noxious weed under the Canada Seeds Act (Woo et al. 1991). It is a difficult weed to control due to its competitiveness and natural tolerance to herbicides, most notably the post-emergence herbicides after the 4-leaf stage (Peng et al. 2005; Peng et al. 2000). After being introduced to Canada from Europe 100 years ago, this weed has been a very adaptable species on the Canadian prairies (McClay and De Clerck-Floate 1999). Heavy infestation can cause substantial crop yield losses (Douglas et al. 1991), and it is also problematic along municipal roads, railroads, and highways.

Researchers at Agriculture and Agri-Food Canada (AAFC) in Saskatoon identified a group of fungi (*Colletotrichum* Corda. spp.) with potential for biocontrol because they selectively attack scentless chamomile. Many species within this genus are host-specific plant pathogens with diverse pathogenic strategies, ranging from intracellular hemibiotrophy to subcuticular/intramural necrotrophy (Bailey et al. 1992; O'Connell et al. 2000; Skipp et al. 1995).

The isolate 00-3B1, identified as *Colletotrichum truncatum,* shows particular promise for biocontrol of scentless chamomile, with consistent host-specific efficacy against the weed. However, one of the major constraints in the development of this bioherbicide is a low efficiency of spore production using agar media. In order to realistically evaluate the potential of this agent for commercialization, it is imperative to develop a system that can be readily scaled up to produce fungal spores efficiently. Although initial methods using complex agar media met preliminary research needs, the process would not be practical or economical for large-scale productions. Submerged fermentation is a promising system for mass production of other bioherbicides (Boyetchko and Peng 2004; Jackson et al. 1996). A range of strategies can be used to manipulate the physical, environmental, and nutritional conditions under submerged fermentation for increased production efficiency as well as optimal bioherbicide fitness.

HYPOTHESIS

Submerged fermentation can be developed to optimize the spore yield and efficacy of *C. truncatum* as a biocontrol agent of scentless chamomile through manipulation of physical, environmental, and nutritional parameters.

OBJECTIVES

The objective of this study was to develop a practical submerged fermentation process for efficient mass production of *C. truncatum*. Specifically:

- To assess the impact of physical and environmental conditions on sporulation of the fungus during submerged fermentation.
- To evaluate the effect of various carbon (C) and nitrogen (N) sources, C concentrations, and C:N ratios in the liquid medium on yield and efficacy of the fungal spore inoculum.
- To validate the feasibility of scaling up production using identified optimal conditions.

STRATEGIES

- Determine the possibility of using a defined liquid medium to replace complex solid media and determine a suitable culturing temperature.
- Evaluate different C and N sources in the selected liquid medium for optimal spore yield and weed control efficacy of the inoculum.
- Using selected C and N sources, evaluate C concentrations and C:N ratios for optimal spore yield and weed control efficacy of the inoculum.
- Using the selected medium composition, evaluate the effect of physical and environmental conditions on sporulation of the fungus.
- Investigate interactions of the most noteworthy nutritional and environmental parameters for further enhancement of spore yield.
- Using the selected medium composition, and physical and environmental conditions, validate the feasibility of scale-up with 20-L fermentors.

2. LITERATURE REVIEW

2.1. *Colletotrichum* spp.

Colletotrichum spp. are prominent in temperate and tropical environments and are arguably one of the most important groups of plant pathogens. Many *Colletotrichum* species cause plant diseases commonly known as anthracnose on stems, leaves and fruits of a range of economically important plants, including cereal crops, grasses, legumes, fruits and vegetables, and a variety of perennials.

2.1.1. Biology of *Colletotrichum* spp.

The genus *Colletotrichum* Corda. belongs to a group of fungi known as Coelomycetes, which is a form-class of Fungi Imperfecti. Coelomycetes produce asexual spores (conidia) in specialized fruiting bodies, usually in the form of either pycnidia or acervuli. Acervuli consist of a cushion of fertile hyphae on the surface of the conidiomata, and may be associated with thick-walled, darkly-pigmented, pointy sterile hyphae known as setae. Acervuli produced by *Colletotrichum* spp. are disc-shaped, waxy, and subepidermal (Bailey et al. 1992; Barnett and Hunter 1998; Cano et al. 2004). During infection, acervuli form in the outer layers of host tissue as anthracnose lesions mature, and eventually erupt to expose conidiophores (Skipp et al. 1995). *Colletotrichum* conidiophores are simple and elongate; conidia are hyaline, 1-celled, ovoid or oblong to falcate (Barnett and Hunter 1998). Characteristics of the conidia are often used to divide *Colletotrichum* species into 2 groups: those with straight conidia and those with falcate conidia (Skipp et al. 1995). Germinating primary conidia may produce secondary conidia, which are generally smaller and less consistent in shape, especially in older cultures (Cannon et al. 2000). Conidia are usually produced in masses inside a mucilaginous matrix composed of glycoproteins, polysaccharides, enzymes, and other constituents (Bailey et al. 1992; Jamil and Nicholson 1989; Skipp et al. 1995). This matrix functions to protect viability of spores under low humidity and to prevent premature germination during inoculum distribution (Bailey et al. 1992).

Sexual reproduction occurs infrequently or not at all (Cannon et al. 2000), but where applicable the teleomorph is known as *Glomerella* (Barnett and Hunter 1998; Sutton 1992), which is classified as an ascomycete (Skipp et al. 1995).

Infection of plant tissues involves multiple steps to overcome the plant's structural barriers both physically and enzymatically, with additional strategies to release phytotoxins or avoid or neutralize certain chemical defences of the plant (Bailey et al. 1992; Skipp et al. 1995). Most *Colletotrichum* species produce undifferentiated germ tubes or appressoria during host penetration (Bailey et al. 1992; Cano et al. 2004; O'Connell et al. 2000; Skipp et al. 1995). Appressoria are thick-walled, melanized swellings formed at the end of a hypha or germ tube to assist with adherence and penetration of host surface tissues. Other specialized infection structures include penetration pegs, infection vesicles, primary hyphae, and secondary hyphae (Bailey et al. 1992; Goodwin 2001; O'Connell et al. 2000). With these mechanisms, *Colletotrichum* species are able to employ diverse pathogenic strategies, ranging from intracellular hemibiotrophy to subcuticular/intramural necrotrophy (Bailey et al. 1992; O'Connell et al. 2000; Skipp et al. 1995). Potentially resistant hosts may defend themselves against the infection though lesion limitation, hypersensitive response, and several other post-penetration resistance mechanisms (Skipp et al. 1995).

Species within this genus historically were classified according to conidial morphology and host range; however this resulted in a deceivingly high number of species (Skipp et al. 1995). Taxonomy within this genus has been revised repeatedly over the last 50 years, during which time about 40 species have been described based on morphology, cultural characteristics, and pathogenicity (Cannon et al. 2000; Sutton 1992). Molecular techniques have also been be used to clarify taxonomic issues (Ford et al. 2004; Skipp et al. 1995; Sreenivasaprasad et al. 1996).

2.1.2. *Colletotrichum truncatum*

Sutton (1992) provided an overview of cultural observations descriptive of *Colletotrichum truncatum* (Schwein) Andrus & W.D. Moore as follows. Colonies are cottony to floccose, pale mouse grey to mouse grey or salmon, ochreceous to cinnamon, submerged to slightly appressed, with an even to irregular margin. Setae are sparse.

Sclerotia are scattered, submerged, irregular, and sometimes confluent. Appressoria are abundant, clavate to irregular, and are sized 6-12 x 6-12 μm. Conidia are formed in saffron to orange masses, and are falcate but not strongly so, fusiform, tapered gradually towards the obtuse apex but abruptly towards the truncate base, and are sized 12-16 x 4-6 μm.

Conidia of *C. truncatum* are capable of germinating on solid substrates lacking nutritional supplements, and therefore are 'nutrient-independent' and likely use endogenous nutritional reserves to facilitate germination (Jackson and Slininger 1993). Analysis of a strain of *C. truncatum* conidia showed endogenous glucose, trehalose, and polyol content is affected by production nutrition such as C:N ratio (Montazeri et al. 2003). Like other *Colletotrichum* species, *C. truncatum* conidia are also surrounded by extracellular materials (Montazeri and Greaves 2002; Montazeri et al. 2003). This fungus requires nutrients for germination in agitated liquid culture, which may be attributed to loss of leaked amino acids into the aqueous environment without the protection of the water-soluble mucilaginous matrix that inhibits diffusion of nutrients on solid media and host plant surfaces (Jackson and Slininger 1993). Transmission electron microscopy examination showed that extracellular materials are not visible on conidia produced in liquid culture but extracellular carbohydrates and proteins were detectable on germinating conidia using cytochemical analysis (Montazeri et al. 2003). The lack of extracellular materials around ungerminated conidia may result in decreased dessication tolerance compared to conidia produced on solid media (Montazeri et al. 2003). Studies with other fungi have similarly demonstrated that conidia produced in liquid culture were less stable in storage formulations compared to conidia produced on solid media, presumably due to physiological differences (Amsellem et al. 1999).

Different strains of *C. truncatum* have been reported to infect and/or cause anthracnose on a wide range of plants belonging to the genera *Lupinus* L.*, Lathyrus* L.*, Lens* P. Mill.*, Pisum* L.*, Vicia* L.*, Indigofera* L.*,* and *Cicer* L. (Weidemann et al. 1988), as well as soybean (*Glycine max* (L.) Merr.) (Tiffany and Gilman 1954), alfalfa (*Medicago sativa* L.) (Graham et al. 1976), cowpea (*Vigna unguiculata* (L.) Walp.) (Adebitan et al. 1996) and several dicotyledonous weeds (Hartman et al. 1986). Five species of *Colletotrichum* have been reported to cause infections in humans, including

C. dematium (Pers.) Grove, a species with such morphological similarity to *C. truncatum* that it has been difficult to confirm which of the two is clinically significant (Cano et al. 2004).

2.2. Scentless chamomile

2.2.1. Biology of scentless chamomile

Scentless chamomile (*Matricaria perforata* Mérat.) is designated by several common synonyms, including wild daisy, scentless mayweed, false mayweed, false chamomile, wild chamomile, corn feverfew, bachelor's button, Kandahar daisy, barnyard daisy, (Graham 2004; Royer and Dickinson 1999; Woo et al. 1991). Scentless chamomile is a native plant of northern and central Europe. It was introduced to Canada at the beginning of last century and is now found across Canada, predominantly in Atlantic and prairie regions, and has been legislated as a noxious weed in Alberta, Saskatchewan, Manitoba, and part of British Columbia (McClay and De Clerck-Floate 1999). It is now designated as a Class 3 secondary noxious weed under the Canada Seeds Act (Woo et al. 1991). Scentless chamomile grows as an annual, biennial, or short-lived perennial plant with white flowers and finely branched leaves belonging to the Asteraceae family. It is an aggressive competitor in annual and perennial crops, forages, non-cropland areas such as roadsides and ditches, and more recently, cultivated crops (Blackshaw and Harker 1997; Royer and Dickinson 1999; Woo et al. 1991).

2.2.2. Control of scentless chamomile

Substantial yield losses may occur when scentless chamomile populations infest crops (Douglas et al. 1991), and therefore control may become crucial from an economic standpoint. Scentless chamomile may be controlled culturally by tilling (Woo et al. 1991), or chemically with spring herbicide application (Blackshaw and Harker 1997). On the Canadian prairies, increasing popularity of conservation tillage has likely contributed to increased prevalence of the weed in cropland (Blackshaw and Harker 1997) and scentless chamomile has a high level of tolerance to many common herbicides used in western Canada (Bowes et al. 1994; Peng et al. 2005), which makes effective weed control a challenge.

2.3. Biological control of weeds

2.3.1. Biocontrol strategies

Integrated pest management (IPM) utilizes a range of options such as crop rotation, mechanical methods, chemical pesticides, and biological control (biocontrol) to maintain crop health by minimizing damage from diseases, insects and weeds. Although crop rotation is the most common cultural method used to keep pests below threshold injury levels, this practice is often underused (Cook 2000). Alternative control methods are limited, and there are certain weeds and agronomic circumstances under which no appropriate chemical herbicides are available and mechanical control is not suitable (Auld and Morin 1995). Some consumers prefer use of non-chemical alternatives in food production, and governments have been lobbied to institute mandates for reducing chemical pesticide usage (Charudattan 2001). Circumstances such as these have motivated research into novel weed control methods such as biocontrol, targeting weeds that lack effective chemical options or situations where current herbicides are at risk of being lost and are not expected to be replaced with new chemicals (Charudattan 1991).

Biocontrol is a method of suppressing pest populations using naturally occurring organisms. Biocontrol agents are generally limited to living portions of an organism that have the ability to infect and limit or kill a target pest (Auld and Morin 1995; Stowell 1991), but the definition may be narrowed to define only microorganisms or broadened to include a plant's natural or modified ability to defend itself (Cook 2000). Four main strategies of biocontrol may be considered as classical, inoculation, inundation, and conservation biocontrol (Eilenberg et al. 2001) and may include augmentation strategies (Charudattan 2001).

Classical and inoculative biocontrol are ecological pest-control methods that involve importation and release of self-disseminating living organisms that generally regulate rather than eradicate a target pest population over the long-term (Boyetchko et al. 2002) in habitats lacking natural enemies (Watson 1991) or without natural resistance to pests (Boyette et al. 1991). Weeds lose their competitive edge and populations are consequently reduced, remaining in equilibrium due to pressure exerted by the control agent population (Mortensen 1986).

Inundative and augmentative biocontrol methods involve the artificial short-term population increase of a living organism in order to suppress or eradicate a target pest population. Augmentation involves maintenance of inoculum sources, by dispersing inoculum at a climatically suitable time to promote endemics by causing infection, disease development, and eventual death of a specific weed host (Charudattan 2001). There is usually an anticipated need for annual repeated applications (Charudattan 1991; Gressel 2003; Mortensen 1986; Silman and Nelsen 1993; Watson et al. 2000).

Regardless of the strategy employed (classical or inundative), biocontrol may be used to control weeds in a variety of agro-ecosystems. This may include crops and crop lands, managed and native pastures or rangelands, plantations, agroforests, and waterways associated with irrigation (Charudattan 2001; Templeton 1992), irrigated crop production, tropical environments, gardens, turf (Auld and Morin 1995) microplots, orchards, and greenhouses (Stack et al. 1988).

2.3.2. Development of bioherbicides

The term 'bioherbicides' is often used when referring to the inundative biocontrol of weeds (Boyetchko et al. 2002; Crump et al. 1999; Eilenberg et al. 2001; Jackson et al. 1996). When the active ingredient is fungal spores or mycelium, the term mycoherbicide may be used (Auld and Morin 1995; Boyette et al. 1991; Charudattan 1991; Crump et al. 1999; Figliola et al. 1988; Templeton 1992; Winder and Van Dyke 1990; Yu et al. 1998). Some authors caution that while bioherbicide and mycoherbicide are the dominant terms used in microbial weed control, the 'cide' suffix should not necessarily be interpreted as implication of death of target weeds (Crump et al. 1999; Eilenberg et al. 2001). Successful microbial biocontrol strategies in the past have returned weeds to their original ecological context rather than providing agronomic control (Gressel 2003), which is sufficient as long as weeds are suppressed long enough to improve crop yields, even if the weeds are not killed (Auld and Morin 1995). *Phytopthora palmivora* Butler (DeVine®), for biocontrol of stranglervine (*Morrenia odorata* Lindl.), was the first fungus to be marketed as a mycoherbicide (Boyetchko et al. 2002; Boyette et al. 1991; Charudattan 1991; Wolken et al. 2003). Despite the huge potential and some early successes, unrealistic expectations of equivalent kill, shelf-life,

and pricing competitiveness of bioherbicides compared to chemical herbicides have partly led to the downfall of development and eventual abandonment of some candidate bioherbicides (Watson et al. 2000), and inundative biocontrol has not yet been adopted by farmers on a large scale (Gressel 2003).

The active ingredients of bioherbicides are generally considered to be the infective living propagules contained in the biocontrol agent formulation (Auld and Morin 1995; Boyetchko et al. 2002). Typically they are native pathogens of the target weed (Cook 2000; Watson et al. 2000), but an exotic species would also be acceptable if it was capable of inciting an endemic disease (Charudattan 1991; Mortensen 1986). For field applications, propagules must be smaller than 100 μm in order to be applied with common spray equipment (Stowell 1991), and depending on the type of organism, may be comprised of bacterial cells or spores, fungal spores (eg. conidia, chlamydospores, blastospores, ascospores), sclerotia, or mycelial fragments (Amsellem et al. 1999; Charudattan 1991; Jackson 1997; Jackson et al. 1996; Pascual et al. 1997; Winder and Van Dyke 1990), as well as protozoa and viruses (Jackson et al. 2004). Over the years, experience has shown that successful bioherbicides must be capable of causing a high level of disease in target plants with specificity towards a single weed species (Chandramohan and Charudattan 2001; Jackson 1997; Jackson et al. 1996). Candidate weeds for biocontrol include those not sufficiently or cost-effectively controlled by herbicides, those for which suitable chemical herbicides do not exist or are expected to become ineffective and new development is not anticipated, and those that have developed resistance or require high rates of chemical, as well as urban allergenic or narcotic weeds (Gressel 2003).

Bioherbicide commercialization involves many of the same considerations as conventional chemical herbicides, such as market stability and size, patent issues, and cost of expanding a basic research project into a commercial venture (Auld and Morin 1995). Bioherbicides are potential candidates for replacement or reduction of chemical herbicides in IPM systems (Charudattan 2001; Mortensen 1986; Winder and Van Dyke 1990 ; Yu et al. 1998). They are biodegradable, do not vaporize, and contamination is benign (Auld and Morin 1995). Because application methods are often similar to chemical herbicides (Auld 1992), they are subjected to similar governmental regulations

(Auld and Morin 1995; Charudattan 1991). The amounts of chemicals applied in an ecosystem may be reduced by combining them with bioherbicides for increased efficacy (Auld and Morin 1995; Boyetchko et al. 2002; Boyette et al. 1991; Charudattan 1991; Graham 2004; Gressel 2003; Peng et al. 2005). Multiple pathogens and/or insect-pathogen combinations may also be applied for synergistic outcomes; increasing the spectrum of weed control, providing insurance against failure of one pathogen under certain conditions and reducing risk of resistance development (Boyetchko et al. 2002; Chandramohan and Charudattan 2001; Gressel 2003). In addition, microbial weed control may be enhanced by facilitating pathogen entry through biological, physical, or chemical wounding (Gressel 2003).

Since the 1950's, fungi have been prevalent in weed biocontrol research, and development of commercial bioherbicides has occurred since the early 1980's (Auld 1992; Charudattan 2001). Fungi are considered advantageous over other microorganisms because many are capable of developing epidemics, infection does not require a damaged or compromised host, and spores are relatively stable (Jackson 1997).

Constraints in development of bioherbicides and other biocontrol agents include biological limitations in disease development, weed competition and resistance, genetic factors, overcoming host defences and variability, host range and specificity, and inconsistent efficacy under varying field conditions (Auld and Morin 1995; Charudattan 1991; Jackson et al. 2004; Jackson and Schisler 1992; Watson et al. 2000). There also may be technological challenges associated with production, shelf-life, formulation, and application, and issues with regulation, profitability, and market competition with chemical pesticides (Auld and Morin 1995; Charudattan 1991; Jackson et al. 2004; Jackson and Schisler 1992; Watson et al. 2000). Some of the most common technological constraints are associated with biological mass production. Because most bioherbicides require a high concentration or volume of propagules to be effective in field application (Morin et al. 1990), failure to produce inoculum on a large scale has hindered the development of some potential bioherbicides (Auld 1992). Therefore, bioherbicide candidates must be suitable for mass production, and propagules produced on this scale must be viable, highly efficacious, genetically stable, and suitable for long-term storage and practical application under a wide range of field conditions (Auld and

Morin 1995; Im et al. 1988; Jackson 1997; Jackson et al. 1996; Pascual et al. 1997; Patino-Vera et al. 2005; Stowell 1991; Walker and Riley 1982; Zhang et al. 2005). Involvement of private industry is often important for this stage of commercial development (Mortensen 1986).

2.3.3. Biocontrol using *Colletotrichum* spp.

Colletotrichum species are well known for crop diseases, but they are also capable of causing disease on some weeds and have become popular bioherbicide candidates for several reasons. *Colletotrichum* is relatively ubiquitous in distribution and strains can be extremely host-specific (Watson et al. 2000). Many *Colletotrichum* species are highly virulent with minimal risk of long-range dissemination (spores dispersal occurs through splashing rain) or persistence in the environment (Templeton 1992). Anthracnose diseases cause rapid and destructive blighting (Skipp et al. 1995), and the hemibiotrophic infection makes these fungi a uniquely specific group of candidates for bioherbicides against annual weeds in annual crops (Goodwin 2001; Templeton 1992; Watson et al. 2000). This type of infection involves a biotrophic phase providing high host specificity followed by a necrotrophic phase that causes extensive tissue damage (Goodwin 2001). The hemibiotrophic nature also allows these organisms to be cultured on synthetic substrates, unlike purely biotrophic microbes, which require a living host to grow and reproduce. *Colletotrichum* strains are important model systems in bioherbicide research and are expected to have the ability to meet some of critical agronomic needs once constraints are overcome (Watson et al. 2000).

Early attention in bioherbicide research was focused on *Colletotrichum* spp. because of the early success and commercialization of Collego® (*C. gloeosporioides* (Penz.) Sacc. f.sp. *aeschynomene*) , as well as Lubao® (*C. gloeosporiodes* f.sp. *cuscutae*) and the prospective commercial products BioMal® (*C. gloeosporiodes* f.sp. *malvae*), Velgo® (*C. coccodes* (Wallr.) Hughes), burr anthracnose (*C. orbiculare* (Berk. and Mont.) Von Arx, DAR), and at least nineteen other potential *Colletotrichum* strains, including *C. truncatum* (Charudattan 1991; Jackson and Bothast 1990; Templeton 1992; Watson et al. 2000). Charudattan (1991; 2001) listed a total of seventeen mycoherbicide projects of interest in 1991 and thirteen examples in 2001 that involved *Colletotrichum*

species. Templeton (1992) outlined eight *Colletotrichum* species considered inadequate as mycoherbicides and seven promising species that were orphaned due to problems with commercial production. In 2002, Boyetchko (2002) listed eight examples of inundative biocontrol agents using *Colletotrichum* species.

2.3.4. Biocontrol of scentless chamomile using *C. truncatum*

A few strains of *C. truncatum* have been investigated as bioherbicide candidates on hemp sesbania (*Sesbania exaltata* (Raf.) Cory) (Boyette et al. 1993; Jackson and Bothast 1990; Silman and Nelsen 1993), purple loosestrife (*Lythrum salicaria* L.) (Nyvall and Hu 1997) and Florida beggarweed (*Desmodium tortuosum* (Sw.) DC.) (Caulder and Stowell 1988).

Due to the natural tolerance to most existing herbicides and a niche market size (Peng et al. 2005), scentless chamomile may be an ideal candidate for consideration of a bioherbicide strategy.

Since 2000, 706 indigenous and exotic fungal isolates obtained through field surveys in Canada and Europe have been tested for biocontrol of scentless chamomile (Peng et al. 2005). Several isolates that were identified as *C. truncatum* showed moderate to high virulence against scentless chamomile without posing negative effects on prairie crops, and were therefore considered suitable candidates for further investigation as a bioherbicide agent (Peng et al. 2005). In several studies, older plants were found to be more resistant to the fungus, but combinations with the herbicides Metribuzin or Clopyralid showed synergy and increased effectiveness compared to either component applied alone (Graham 2004; Peng et al. 2005).

2.3.5. Production of *Colletotrichum* spp. as bioherbicides

Mass production of *Colletotrichum* spp. has been achieved in commercial scale facilities (Templeton 1992). Collego®, a product for control of northern joint vetch (*Aeschynomene virginica* (L.) BSP.), was produced using liquid fermentation (Charudattan 1991), and while early attempts in custom fermentors were not cost-effective (Watson et al. 2000), Collego® was later made available commercially (Boyetchko et al. 2002; Gressel 2003). BioMal® was promising for control of round-leaved mallow (*Malva rotundifolia* L.), and the development was near

commercialization by 1991 (Boyette et al. 1991; Charudattan 1991). In 1992, it became the first bioherbicide registered in Canada (Boyetchko et al. 2002), however, marketing was not pursued. Charudattan (1991) described the organism as 'easily cultured' in reference to a personal communication with Mortensen, but later reports indicated the inability to produce BioMal® cost effectively was part of the marketing delay (Boyetchko et al. 2002; Charudattan 2001; Watson et al. 2000). Velgo® was investigated as a dry formulation for post-emergent control of velvetleaf (*Abutilon theophrasti* Medic.) (Templeton 1992; Yu et al. 1998). Liquid culture fermentation was used to produce this agent for greenhouse and field testing (Yu et al. 1998). Yield optimization was investigated to increase the scale of the liquid production system (Watson et al. 2000) and commercial development was reported to have been attempted (Boyetchko et al. 2002). Burr anthracnose underwent development for use as a post-emergent biocontrol agent of spiny cocklebur (*Xanthium spinosum* L.) (Templeton 1992), but failed to become a commercial product (Boyetchko et al. 2002), partly due to low spore yields in submerged culture during scale-up (Watson et al. 2000).

Development of cost-effective methods of spore production of *C. truncatum* for biocontrol of hemp sesbania was required because this fungus was not amenable to submerged culture sporulation with traditional liquid culture media (Jackson 1997; Jackson and Bothast 1990). For this reason, a defined medium that supported growth and sporulation of *C. truncatum* was developed (Jackson 1997; Jackson and Bothast 1990), followed by investigation of optimal cultural conditions to maximize sporulation and efficacy (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson and Slininger 1993). Production of conidia and microsclerotia could be induced by manipulating nutrition of the medium, and it was found that microsclerotia were more effective than conidia for biocontrol of hemp sesbania (Jackson 1997).

Many resources have been devoted to developing production procedures and some scientists believe that amenability to liquid production should be an earlier screening factor for potential biocontrol agents worthy of additional evaluations (Vega et al. 2003). However, it may not be fully justified to ignore all poor sporulators during screening, as sporulation may be improved or sometimes alternative formulations can be developed using vegetative fungal mycelium (Amsellem et al. 1999). From a practical standpoint,

it is useful to develop standard production protocols early on for biological evaluation, because not all production conditions foster the same level of fitness in the final propagules (Jackson et al. 1996).

2.4. Fermentation of biopesticides

Böck (2000) defined fermentation as an anaerobic type of metabolism in which an organism produces a mixture of oxidized and reduced compounds through degradation of organic compounds in the absence of external electron acceptors, while Hilton (1999) referred to fermentation simply as the aerobic and anaerobic cultivation of microorganisms. Industrial microbiologists consider fermentation to be any process that involves mass culturing of a microorganism, resulting in a desired product (Stanbury et al. 1995c).

Basic features of any fermentation include formulation of culturing media, adequate sterilization of media and supplies, production of pure and viable inoculum culture, fermentation of an organism according to optimal growth conditions, and finally extraction and purification of desired product and disposal of effluent.

2.4.1. Solid fermentation

Solid media are often used for primary screening of potentially useful microorganisms (Davis and Blevins 1979), and for early biopesticide production because most fungi can sporulate on appropriate agar media (Jackson 1997). Inoculum yields achieved using these methods often are sufficient for initial testing or for inoculation of liquid media (Jackson 1997), but media often contain expensive ingredients that may not be suitable for large-scale production (Yu et al. 1998).

It is often challenging to sterilize, inoculate, and store the materials used for solid fermentation (Boyette et al. 1991) and the processes may be more prone to contamination (Jackson 1997; Morin et al. 1990). Some fungi grow slowly on solid cultures and are more easily out-competed by contaminants (Urquhart et al. 1994). There may also be problems with gas exchange, temperature control, and product recovery (Jackson 1997). Problems with propagule recovery may result in the substrate becoming part of the end product, negatively affecting the quality of bioherbicides (Auld and Morin 1995). Fermentation times are usually longer on solid substrates compared to

liquid, thereby increasing production costs (Jackson 1997). While most fungi will sporulate readily on suitable solid media, a large surface area is required to generate a sufficient amount of spores for mass production (Stanbury et al. 1995b), and this space may not be available or practical. Solid-substrate production is often more time-consuming and labour intensive than other methods (Boyette et al. 1991; Morin et al. 1990), unless located in a part of the world where labour is less costly and suitable raw material is readily available (Auld 1992; 1993; Jackson 1997). Overall, most solid substrates are considered too expensive for commercial use and further cost-saving developments may be required before these methods can be considered commercially attractive (Jackson et al. 1996).

2.4.2. Submerged fermentation

Submerged liquid culturing is usually preferred for large-scale fermentations (Davis and Blevins 1979), and has been used extensively for industrial production of antibiotics, amino acids, ethanol, organic acids, baker's and distiller's yeasts. This technological experience provides a critical knowledge base as well as industry acceptability for production of bioherbicides using similar methodology (Jackson 1997; Jackson et al. 1996). However, protocols may not yet be refined enough to produce viable and highly efficacious spores for filamentous fungi (Auld and Morin 1995). While many fungi may not sporulate well in submerged cultures (Charudattan 2001; Thomas et al. 1987), there is evidence that most non-fastidious plant pathogenic fungi can be produced using submerged fermentation (Stowell 1991). Some fungi are even seemingly restricted to submerged cultures for production of conidia, requiring high moisture contents or aeration levels (Ogel et al. 1994).

Submerged fermentation is considered more readily available, economical, and practical than other methods for mass production of biopesticides in developed countries (Auld 1992; 1993; Auld and Morin 1995; Stowell 1991; Yu et al. 1997). It is generally believed that liquid fermentation is preferred or required to produce low-cost bioherbicide agents (Charudattan 1991; 2001; Jackson 1997; Jackson et al. 1996; Templeton 1992; Watson et al. 2000). There are several reasons for this. One-stage submerged cultivation can produce spores faster than other methods (Thomas et al.

1987). Some existing equipment (flasks or bioreactors) can be utilized without modification (Auld 1993). Liquid cultures are homogenous, which makes them easier to control, maintain, and monitor (Jackson 1997). Propagules produced in submerged fermentation are usually relatively easy to recover using centrifugation or filtration methods (Davis and Blevins 1979), which are generally more efficient than most harvest techniques used in solid-substrate productions (Auld and Morin 1995). Submerged cultures are generally easier to operate aseptically compared to solid media and may be more readily applied on a large scale (Stanbury et al. 1995b). These benefits may translate into lower production costs for bioherbicide propagules (Jackson 1997). Collego® and DeVine® have been produced using submerged fermentation (Auld 1992; 1993; Boyetchko et al. 2002; Boyette et al. 1991; Stowell 1991; Templeton 1992).

Batch fermentation outputs generally follow a sigmoidal curve that includes lag, exponential, stationary, and eventually decline phases (Auld 1992; 1993). Microorganisms are inherently unpredictable and therefore media and process development can constantly present new challenges despite researchers' previous fermentation experience. Even closely related organisms may have vastly different nutritional and environmental requirements for growth, survival, virulence, and metabolite production (Stack et al. 1988; Stowell 1991). As a result, production systems for bioherbicides often need to be developed empirically and intelligently; some claim it is as much an art as a science (Charudattan 2001; Dahod 1999; Hilton 1999).

2.4.3. Inoculum characteristics

In order to promote success in fermentation, an appropriate volume or concentration of inoculum should be available in a healthy and active state, in a suitable morphological form, free of contaminants, and capable of forming the desired product (Stanbury et al. 1995b), which in the case of bioherbicides would be efficacious propagules, as opposed to a secondary by-product often desired in conventional industrial fermentations (Stowell 1991). Inoculum for submerged cultures should provide a phenotypically consistent microbial mass at an appropriate time for a relatively low cost (Monaghan et al. 1999). For fermentation inoculum consisting of conidia, each spore is a colony forming unit (CFU), whereas mycelial biomass is more

difficult to quantify (Amsellem et al. 1999). Loss of virulence may also occur through sub-culturing, therefore repeated transfer of cultures should be avoided (Auld 1992; Van Den Boogert 1989).

Initial inoculum concentration can affect fermentation productivity (Monaghan et al. 1999), and 1-15% (v/v) is frequently used. The morphological form of an organism in liquid culture may be affected by initial inoculum concentration (Stanbury et al. 1995b). High concentrations of spore inoculum may result in a dispersed form of growth, while low concentrations may result in pellet formation. Pellets are compact, discrete masses of hyphae, while dispersed forms are filamentous, homogenous suspensions in the media [M.D. de Jong, C.J.E.A. Bulder, C.A.G.M. Weijers, P.C. Scheepens (unpublished work), 1982; Stanbury et al. 1995b]. In addition, fungi in liquid culture may also take the form of small discrete cells or larger floccose pellets (Auld 1993). Pellets often do not grow exponentially and are not dispersed homogeneously (M.D. de Jong, C.J.E.A. Bulder, C.A.G.M. Weijers, P.C. Scheepens (unpublished work, 1982)), and pelleted mycelium may not be as productive if the centre of the pellet is starved of nutrients (Stanbury et al. 1995b). Cultures growing in large colonies as clumps, balls, or pellets, may be at a disadvantage because this pattern may result in limited liquid-solid mass transfer (K_La) rates, a symptom of poor mixing (Hilton 1999). Production of vegetative cells or mycelium may accompany fungal sporulation, but this actually lowers spore biomass yield on a per gram of substrate basis (Wolken et al. 2003).

A range of initial inoculum concentrations has been cited in literature when describing fungal submerged fermentation (de la Torre and Cardenas Cota 1996; Li and Holdom 1995; Thomas et al. 1987; Urquhart et al. 1994; Vega et al. 2003; Vidal et al. 1998; Zhang et al. 2001). There was a negative relationship between seed culture inoculum density and conidia yield in liquid fermentation of *Phomopsis convolvulus* Ormeno (Morin et al. 1990). While an initital inoculum concentration of 5×10^5 sp/ml was used in pre-production flask cultures, a higher concentration of blastospores at $1\text{-}5 \times 10^6$ sp/ml was used to promote competition of *Paecilomyces fumosoroseus* (Wize) A.H.S. Br. & G. Sm. against contaminants in non-sterile portable fermentation equipment (Jackson et al. 2004).

2.4.4. Media selection and nutritional conditions

Medium formulation is the crucial first step in developing successful fermentation processes (Stanbury et al. 1995a). As the major component of all fermentation media, a large quantity of clean water is required, preferably of consistent composition from a reliable source (Stanbury et al. 1995a), and water impurities may also become part of the medium (Dahod 1999). Other basic components usually include inorganic salts, C sources, N sources, vitamins, minerals, and access to oxygen (Auld 1993; Dahod 1999; Davis and Blevins 1979; Hilton 1999; Stanbury et al. 1995a). Media must contain ingredients such as carbohydrates, lipids, proteins, and elements that meet the basic needs of the microorganism of interest, providing an adequate supply of energy for biosynthesis and cell maintenance (Hilton 1999; Stanbury et al. 1995a).

Defined or synthetic media contain a known and easily altered quality and quantity of each ingredient, and semi-synthetic has few unknown components, while complex media usually contain crude ingredients and composition is not known in detail (Davis and Blevins 1979). The first step in optimizing fermentation is to develop a defined medium that supports satisfactory growth and propagule formation (Jackson 1997; Jackson et al. 1996). Selection of any fermentation ingredients involves the balancing of several important criteria: effect on total biomass or product yield, amount produced per gram of substrate used, minimal undesired by-product formation, availability of medium components with consistent quality and suitability for downstream processing (Stanbury et al. 1995a). Sources of C and N, trace metals, vitamins, C concentration, and C:N ratio have been reported to affect growth, propagule formation, and efficacy of bioherbicide agents (Jackson 1997; Jackson et al. 1996; Yu et al. 1998; Zhang et al. 2001). Therefore, once a defined medium is developed, these nutritional components may be manipulated to improve these parameters.

Media often contain supplemental minerals, vitamins, and nutrients that a microorganism may not be able to synthesize (Jackson 1997; Jackson et al. 1996; Stanbury et al. 1995a). Minerals are sources of major nutrients, trace metals, ionic strength-balancing agents, secondary metabolite precursors, buffers, pH control agents, and reactants (Dahod 1999). Important trace elements include iron, zinc, copper, manganese, and potassium (Stanbury et al. 1995a; Zabriskie et al. 1980). Vitamins are

complex organic compounds required in small amounts as coenzymes in catalysis during microorganism metabolism (Zabriskie et al. 1980). In general, media require thiamine, niacin, pantothenate, riboflavin, and certain B vitamins in greater amounts, and folic acid, biotin, vitamin B_{12} and lipoic acid in smaller amounts (Zabriskie et al. 1980). Macro and micronutrients have been shown to have an effect on germination, mycelial growth, sporulation and/or efficacy of several *Colletotrichum* species (Bailey et al. 1992; Jackson et al. 1996; Yu et al. 1997).

Knowledge of a specific microorganism's composition is useful to determine the type and quantities of elements required in a growth medium. In general, fungal element composition ranges are (% by dry weight) 40-63% C, 7-10% N, 0.4-4.5% phosphorus, 0.1-0.5% sulphur, 0.2-2.5% potassium, 0.02-0.5% sodium, 0.1-1.4% calcium, 0.1-.05% magnesium, and 0.1-0.2% iron (Stanbury et al. 1995a). For production of biomass and growth-associated products, nutrient-balanced media are typically used (Hilton 1999). Some dimorphic fungi may require optimal nutrition to produce high biomass, but sporulation in liquid cultures is often stimulated by nutritionally poor media for fungi that require a trigger for differentiation of conidia from vegetative growth (Vega et al. 2003). An understanding of nutrition requirements of certain bioherbicides also provides insights into pathogenesis of the product (Goodwin 2001).

In general, the effectiveness of nutrition on fungal growth and sporulation varies depending on the species, and a choice favourable for biomass increase may not necessarily be suitable for optimal sporulation (Li and Holdom 1995). The following sections outline nutritional factors which are of particular interest when designing fermentation media for production of fungal inoculum.

2.4.4.1. Carbon sources

Some fungal conidia, such as *Beauveria bassiana* (Bals.) Vuill, require an utilizable C source to germinate (Thomas et al. 1987) while others, such as *C. truncatum,* are capable of using endogenous nutritional reserves for germination (Jackson and Slininger 1993). Both biosynthesis and energy generation require a C substrate (Stanbury et al. 1995a). Carbohydrates are important C sources in the form of simple sugars or polymers, but they also provide a source of oxygen and hydrogen

(Zabriskie et al. 1980). Virulence and bioherbicide stability may be affected by C source (Stowell 1991).

For an aerobic fermentation the following formula can be used to estimate the C requirement as a cellular yield coefficient *Y* (Stanbury et al. 1995a):

Y = (quantity of cell dry matter produced) (quantity of C substrate used)$^{-1}$

Choice of C source depends on the desired product of fermentation, as well as the availability, quality, and cost of different sources (Stanbury et al. 1995a). Availability of carbohydrates $(CH_2O)_n$ is ranked hexoses > disaccharides > pentoses > polysaccharides (Zabriskie et al. 1980). Molasses, cereal grains, starch, glucose, sucrose, and lactose are generally inexpensive (Stanbury et al. 1995a). Crude agricultural products such as cornstarch, corn flour, glucose, hydrolyzed-corn-derived materials, glycerol, and sucrose are also low cost and available in large quantities (Boyette et al. 1991). Organic acids, such as acetic acid, have also been investigated by the fermentation industry (Dahod 1999). While C may be in the form of oils and fats, hydrocarbons and hydrocarbon derivatives, or carbohydrates, the latter are used most commonly in microbial fermentation processes (Dahod 1999; Stanbury et al. 1995a; Zabriskie et al. 1980).

Glucose or dextrose ($C_6H_{12}O_6$) is a monosaccharide that is one of the most common types of carbohydrates because it is available in monomeric form and can be used by most fungi immediately (Ooijkaas et al. 1998). It is used in the greatest volume by the fermentation industry, and can be obtained from the hydrolysis of corn starch, grains, or other cellulosic materials (Dahod 1999; Zabriskie et al. 1980). Various types of glucose-based carbohydrates are available as more economical C sources but a more expensive dextrose monohydrate is usually used for small-scale applications because of its purity (Dahod 1999). Physiological roles of glucose include regulatory functions, such as regulation of storage compound (eg. trehalose and lipids) accumulation, or induction of signal repression of enzyme systems responsible for breaking down complex molecules (Stowell 1991). This may in turn affect bioherbicide virulence if the enzymes are required for pathogenesis. Using glucose as the C source in a defined medium has resulted in good levels of conidial production for *C. truncatum* in liquid culture (Jackson and Bothast 1990; Montazeri and Greaves 2002). Glucose has been preferred as a C source for sporulation of several other fungal biopesticide species under

submerged fermentation conditions (Cliquet and Jackson 2005; Im et al. 1988; Thomas et al. 1987; Urquhart et al. 1994; Vega et al. 2003; Zhang et al. 2001).

Fructose ($C_6H_{12}O_6$) is a simple sugar that is an isomer of glucose. It can be found in a variety of fruits and vegetables and is also obtained from the breakdown of sucrose. Sucrose ($C_{10}H_{22}O_{11}$) is a dissacharide composed of glucose and fructose. It can be obtained from sugar cane and sugar beet (Stanbury et al. 1995a), and is available in various degrees of refinement (Dahod 1999). Sucrose was favoured for liquid production of *C. coccodes,* three isolates of *C. graminicola* (Ces.) Wills, and *Plectosporium tabacinum* (van Beyma) Palm, Gams & Nirenberg (Jamil and Nicholson 1989; Yu et al. 1997; Zhang et al. 2001).

Glycerol ($C_3H_8O_3$) is a water soluble sugar alcohol that is also known as glycerin or glycerine. Lactose ($C_{12}H_{22}O_{11}$) is a dissacharide composed of glucose and galactose. Gradually metabolized C sources like glycerol and lactose may be selected to slow down vegetative growth in order to increase metabolite production (Dahod 1999; Stanbury et al. 1995a). Lactose is often obtained from cheese whey and is used by the fermentation industry more frequently than maltose, mannitol, sorbitol, and xylose, but not to the same extent as glucose and sucrose (Dahod 1999; Zabriskie et al. 1980). Lactose was frequently used during early fermentations of penicillin (Dahod 1999).

Trehalose ($C_{12}H_{22}O_{11}$) and maltose ($C_{12}H_{22}O_{11}$) are dissacharides composed of two glucose units. Cellulose ($C_6H_{10}O_5$) is a linear polymer of glucose molecules. It is the most plentiful organic compound found on earth, but few organisms can utilize it as a C source (Zabriskie et al. 1980). Polymers such as cellulose or starch must be hydrolysed by the fungus before they can be used (Ooijkaas et al. 1998). Cellulose is insoluble in water due to inter- and intra-chain hydrogen bonds (Zabriskie et al. 1980). Cane molasses is composed of approximately 33.4% (w/v) sucrose and 21.2% invert sugars (Stanbury et al. 1995a). Molasses is categorized into four grades: beet molasses (by-product of table sugar refined from sugar beets), blackstrap molasses (crystallization of brown sugar from sugar cane), refiner's cane molasses (recrystallization of brown sugar to white sugar), and high test molasses (inverted cane syrup) and is a source of carbohydrates as well as N, inorganic constituents, and vitamins (Zabriskie et al. 1980). It has been used as a fermentation carbohydrate in production of ethanol, single cell

protein, organic and amino acids, microbial gums, antibiotics, specialty enzymes, vaccines, and fine chemicals (Stanbury et al. 1995a). Although molasses is a cheap C source, it usually contains impurities.

Depending on the fungal species, C sources that promote fungal sporulation do not necessarily yield highest biomass and vice versa (Boyette et al. 1991). In culturing *Verticillium biguttatum* Gams, glucose, fructose, mannitol, and cellobiose resulted in the most mycelial growth but galactose was superior for conidiation (Van Den Boogert 1989). The TKI broth amended with glycerol produced *B. bassiana* with nearly equal proportions of conidia and blastospores, while glucose, fructose, citrate, and lactose favoured conidium production and maltose, sorbitol, and starch favoured blastospore production (Thomas et al. 1987). Production of hyphal biomass of *Talaromyces flavus* (Klöcker) Stolk & Samson was lowest on monosaccharides and highest on complex sugars such as polysaccharides and β-glucosides, while ascospore yields were highest on oligosaccharides but lower on monosaccharides and polysaccharides (Engelkes et al. 1997). Mycelial growth of *Fusarium moniliforme* var. subglutinans Wollenw. & Reinking was highest on solid media containing maltose, lactose, or soluble starch and most dense on media containing glucose, while sucrose, fructose, or soluble starch supported the highest sporulation (Bolkan et al. 1982). Starch was preferred over glucose for sporulation of *Coniothyrium minitans* Campb. because substrate inhibition occurred when glucose was used (Ooijkaas et al. 1998). A variety of carbohydrates supported growth of two isolates of *Metarhizium anisopliae* (Metschn.) Sorokin, but sorbose did very poorly and soluble starch was the best for growth and sporulation (Li and Holdom 1995).

2.4.4.2. Nitrogen sources

While germination of conidia usually requires a C source, continued growth of fungi may be dependent on both C and N supplies (Thomas et al. 1987). Approximately 10% of the dry weight of organisms may be assumed to be N (Dahod 1999), which is the second most abundant element next to C and therefore is used in the second highest portion in fermentation media (Zabriskie et al. 1980). Organisms need N for metabolic energy, amino acids, purines, pyrimidines, and the polymers created from these

nitrogen-containing cellular substances; namely proteins, DNA, and RNA (Zabriskie et al. 1980). The N source may also influence the virulence and stability of a bioherbicide (Stowell 1991).

Organic N supply often results in faster growth. Organic N may be supplied to microorganisms in the form of amino acids, proteins, or urea (Stanbury et al. 1995a; Zabriskie et al. 1980). Proteins and amino acids also provide C, oxygen, hydrogen, and sulphur. Other relatively inexpensive N sources include ammonium salts, urea, nitrates, corn steep liquor, soybean meal, slaughter-house waste and fermentation residues (Stanbury et al. 1995a). Some N sources for fermentation are derived from agricultural products, brewery industry by-products, or protein-rich meat and fish by-products (Dahod 1999). Complex N sources can be problematic in downstream processing and effluent disposal (Stanbury et al. 1995a). Most industrial microorganisms can utilize organic or inorganic N sources. A few examples below demonstrate their uses in production of fungal biopesticides.

Jackson and Bothast (1990) reported that casamino acids contained 53% C and 16% N, but later corrected their N estimate to 8% (Jackson and Schisler 1992; Jackson and Slininger 1993). In liquid media, complex N sources were generally superior, and media supplemented with casamino acids resulted in the highest conidial yields for a *C. truncatum* strain when compared to 18 other N sources tested, including ammonium sulphate and individual amino acids (Jackson and Bothast 1990). In a defined medium, casamino acids had relatively more consistent composition than proteinacious N sources (Jackson and Bothast 1990), and promoted *C. truncatum* sporulation more effectively than urea and individual amino acids (Jackson and Slininger 1993). Casamino acids have been used as a N source for sporulation of other fungal biopesticide species in submerged fermentation, such as *P. fumosoroseus*, *B. bassiana,* and *M. anisopliae* (Cliquet and Jackson 2005; Jackson et al. 2004; Vega et al. 2003).

L-leucine and L-glutamic acid are amino acids that have both been shown to support sporulation of *C. falcatum* Went, but the former yielded significantly lower spores compared to the latter (Olufolaji 1994). L-glutamic acid is found in vegetables, fruits, dairy products, and meat. A related compound, glutamine, can also be used as a

N source for fungal fermentation (Van Den Boogert 1989). Glutamine is heat sensitive and should not be autoclaved (Li and Holdom 1995).

Ammonium nitrate (NH_4NO_3) is the most commonly used inorganic N source (Zabriskie et al. 1980), as well as a pH control agent in fermentation (Stanbury et al. 1995a). The ammonium ion will be metabolized first, which usually causes an acid drift as a proton is released (Vidal et al. 1998), followed by an alkaline drift as the nitrate is metabolized upon exhaustion of the ammonium ion (Stanbury et al. 1995a). Few organisms are able to assimilate nitrites, but fungi commonly are able to use nitrates such as ammonium nitrate and sodium nitrate (Zabriskie et al. 1980). Ammonium nitrate, ammonium sulphate, sodium nitrate, potassium nitrate and other nitrogen-containing salts have also been combined with organic N for fermentation (Dahod 1999). Nitrate assimilation requires a lot of energy to reduce nitrate to nitrite and then to ammonia (Ooijkaas et al. 1998). Ammonium-nitrogen compounds were suitable for growth and sporulation of *V. biguttatum* but nitrate-nitrogen could not be utilized (Van Den Boogert 1989). Ammonium nitrate resulted in the highest mycelial dry weight in broth production of *Trichoderma harzianum* Rifai, and was second only to ammonium sulphate for best sporulation (Jayaraj and Ramabadran 1998). Potassium nitrate (KNO_3, 14% N) was selected as a N source for production of *B. bassiana* over complex N sources (Thomas et al. 1987). It was shown to be a poor N source for sporulation of *C. capsici* (Syd.) Butler & Bisby but an excellent source for *C. curcumae* (Syd.) Butler & Bisby (Palarpawar and Ghurde 1997). In a different study, without organic N or soy protein, KNO_3 was poor for mycelial growth and sporulation of *C. coccodes* (Yu et al. 1997; Yu et al. 1998). A moderate number of *P. tabacinum* spores was produced in media containing KNO_3, but these spores were more efficacious than those produced with a higher-yielding medium using corn gluten meal as the N source for biocontrol of false cleaver (*Galium spurium* L.) (Zhang et al. 2001).

Cottonseed hydrolysate is a proteinacious organic N source (Stanbury et al. 1995a) that can be used by microorganisms capable of excreting extracellular proteases that hydrolyze proteins to amino acids (Zabriskie et al. 1980). Pharmamedia Cottonseed Hydrolysate consists of 59.20% protein and 24.13% carbohydrates plus other vitamins

and minerals (Zabriskie et al. 1980). Cottonseed flour has been used for penicillin fermentation because the N is slow releasing (Dahod 1999).

Tryptone is a complex N source that is essentially a peptone produced by proteolytic digestion of casein by the protease trypsin, resulting in a mixture of peptides, amino acids, and water-soluble vitamins. It has been shown to be a remarkably good N source for sporulation of *C. capsici* (Palarpawar and Ghurde 1997).

Urea and ammonium sulphate were less favourable for sporulation of *C. truncatum* than other N sources tested (Jackson and Bothast 1990). While urea was the best source of N for *C. curcumae,* it did not support sporulation of *C. capsici* (Palarpawar and Ghurde 1997) or *C. falcatum* (Olufolaji 1994). In contrast, ammonium carbonate, sodium nitrate, and potassium nitrate supported both *C. curcumae* and *C. capsici* (Palarpawar and Ghurde 1997) and spore yields for *C. falcatum* were also increased when organic N sources were used (Olufolaji 1994). Ammonium oxalate, sodium nitrite, or thiourea did not support sporulation of either *C. capsici* or *C. curcumae* (Palarpawar and Ghurde 1997). Either casamino acids or soy protein could be used to replace the more expensive V-8 juice in a modified Richard's medium used to produce *C. coccodes,* without compromising spore yields, while inorganic sources did not support adequate sporulation (Yu et al. 1997). Other biopesticides *P. fumosoroseus, T. flavus, M. anisopliae, C. minitans, B. bassiana, P. tabacinum,* have been shown to prefer different N sources (de la Torre and Cardenas Cota 1996; Engelkes et al. 1997; Li and Holdom 1995; Ooijkaas et al. 1998; Thomas et al. 1987; Vidal et al. 1998; Zhang et al. 2001).

2.4.4.3. Carbon concentration

The rate at which a C source is metabolized can play an important role in biomass formation or metabolite production (Stanbury et al. 1995a). Typically the C source content in liquid media ranges from 0.2-20% (Zabriskie et al. 1980).

Carbon concentration was shown to regulate conidial and microsclerotial formation of the bioherbicide *C. truncatum* (Jackson and Bothast 1990; Jackson et al. 1996). Between 10-30 g/L glucose (5.1-15.3 g C/L), higher yields of conidia were produced. However, when glucose concentration was increased to 50 g/L or higher,

sporulation was inhibited while microsclerotia production was increased (Jackson and Bothast 1990; Jackson et al. 1996). This trend was not observed when similar tests were performed for *C. gloeosporioides* f. sp. *aeschynomene*, for which sporulation continued to increase as C concentration increased (Jackson and Bothast 1990). It was found that regardless of the initial C concentration, glucose was depleted from all cultures of *C. truncatum* after a 7-d fermentation, and it was concluded that C depletion was not essential for sporulation (Jackson and Bothast 1990). Submerged fermentation of *C. truncatum* with optimized levels of glucose (20g/L) indicated that the sugar was consumed within 2 days and maximum spore concentrations were achieved one day after that (Silman and Nelsen 1993). Sporulation of *C. coccodes* increased as sucrose concentration increased, but specific spore yields were constant, indicating that C concentration had little effect on raw material conversion to spores (Yu et al. 1998).

The effect of C concentration has been studied in fermentation of other biopesticides, including *P. fumosoroseus, B. bassiana, M. anisopliae, C. minitans,* and *P. tabacinum,* exhibiting various effects on biomass, sporulation rate and yield, efficacy, and shelf-life (Cliquet and Jackson 2005; Ooijkaas et al. 1998; Vega et al. 2003; Vidal et al. 1998; Zhang et al. 2001).

2.4.4.4. Carbon to nitrogen ratio

A medium with a balanced C:N ratio generally encourages growth of mycelium (Boyette et al. 1991). For fungi, a balanced ratio may be in the range of 4:1 to 9:1 based on their general chemical composition (Stowell 1991). Relative amounts of C and N in a medium available to a fungus may determine the relative amounts of conidia or mycelium produced (Im et al. 1988).

Studies have demonstrated that C:N ratio can affect spore yield of *Colletotrichum* species (Bothast et al. 1993; Jackson and Bothast 1990; Jackson et al. 1996; Montazeri and Greaves 2002; Schisler et al. 1995; Yu et al. 1998) and other fungal biopesticides (de la Torre and Cardenas Cota 1996; Im et al. 1988; Morin et al. 1990; Pascual et al. 1997; Thomas et al. 1987; Zhang et al. 2001). Montazeri and Greaves (2002) found significantly lower sporulation in media with a 40:1 (C:N) ratio for a strain of *C. truncatum* compared to other C:N ratios tested. They also found that *Alternaria*

alternata (Fr.) Keissl. behaved in a similar way, yielding more conidia in media with C:N ratios of 15:1 and 5:1 than that of 40:1. Jackon and Bothast (1990) showed that a medium containing glucose (C) and casamino acids (N) in a ratio of 15:1 produced significantly more *C. truncatum* conidia than media with ratios of 40:1 or 5:1, regardless of the glucose concentration used. Jackson and Schisler (1992) tested C:N ratios from 10:1 to 80:1 for production of *C. truncatum*, and found that after 48 hours amino acids and glucose were depleted from 10:1 and 30:1 media and the rate of biomass accumulation declined when sporulation began. Although amino acids were depleted at a similar rate in the 80:1 medium, glucose was not used up even after 5 days and biomass continued to accumulate even after sporulation began. Sporulation was lower in the 80:1 medium than in 10:1 and 30:1 media, which were not significantly different from each other. Conidia produced in the 10:1 medium had significantly higher protein but lower lipid content than those produced in 30:1 or 80:1 media. Increased protein content was associated with higher spore germination and appressorial formation, and a C:N ratio between 20:1-15:1 with 4 or 8 g C/L was determined to be optimal (Jackson et al. 1996). Silman and Nelsen (1993) developed a similar medium for *C. truncatum* but suggested the optimal C:N ratio to be about 12:1. Further analysis of *C. truncatum* conidia produced in media with different C:N ratios revealed that with an increase of C:N ratio in the medium, glucose contained in the conidia tended to increase but relative amounts of trehalose and total polyols (including glycerol and mannitol) decreased (Montazeri et al. 2003). Medium composition did not affect the extracellular carbohydrates and proteins for these conidia, but spores produced in C limited cultures were more tolerant to desiccation (Montazeri et al. 2003).

C:N ratio was also a key factor for production of *C. coccodes* (Yu et al. 1998), with differing effects on sporulation and mycelial growth depending on the C concentration. At low C concentrations (5-10 g C/L), biomass increased and spore yields decreased with an increase in C:N ratio; at 20 g C/L as C:N ratio increased up to 15:1 biomass decreased but spore yields increased, while above 15:1 an opposite effect was observed and media became viscous after 5 days because N became a limiting factor that caused extra C sources to be converted to spore matrix.

2.4.5. Effect of medium composition on bioherbicide efficacy

Nutritional conditions may also affect the efficacy of spores produced in submerged fermentation. Differences in efficacy of spores produced in different C and N environments may be related to an association between increased protein content and rapid conidial germination (Jackson et al. 1996). Excess amounts of glucose in media may be converted to lipid, thereby reducing the protein content in spores, leading to lower conidial germination and bioherbicidal efficacy.

Media with a C:N ratio of 30:1 consistently produced more *C. truncatum* conidia than those with 10:1 or 80:1 ratios but the lower C:N ratio favoured rapid germination of conidia and formation of appressoria, and inoculum was subsequently more efficacious against hemp sesbania (Bothast et al. 1993; Jackson and Bothast 1990; Jackson et al. 1996; Schisler et al. 1995; Yu et al. 1998). Conidia of *C. truncatum* produced in a medium with a 5:1 ratio and 4 g/L C, showed higher germination and caused more lesions on hemp sesbania, with germination after a 30-day storage higher than those produced at 40:1 (Montazeri and Greaves 2002). Differences in efficacy resulting from varying C:N ratios of the medium may be compensated by certain inoculum treatments or amendments to bioherbicide formulations, such as washing conidia with 0.9% NaCl (Montazeri and Greaves 2002) or adding pregelatinized starch after fermentation to conidial formulations prior to treatment (Bothast et al. 1993; Schisler et al. 1995).

Spores of *C. coccodes* produced in basal media with C:N ratios of 10:1 and 15:1 reduced velvetleaf biomass by a significantly greater degree than spores produced at 5:1 or 20:1 (Yu et al. 1998). Using sucrose and potassium nitrate as C and N sources and a C:N ratio of 15:1, C concentration affected spore concentration of *P. tabacinum* and efficacy against false cleavers (*Galium spurium* L.), with a C concentration of 12.6 g/L producing the highest spore concentration and the greatest biomass reduction of the weed compared to C concentrations between 4.2 to 33.6 g/L, while C:N ratio affected sporulation but not efficacy (Zhang et al. 2001). Media containing sucrose supported moderate sporulation of *P. tabacinum* but spores produced with this C source caused higher biomass reduction of false cleavers at 99.8% compared to galactose, which produced spores profusely but only reduced biomass by 62% (Zhang et al. 2001). Other studies suggest varying impact of C:N on efficacy of different biocontrol inocula

produced using submerged fermentation (de la Torre and Cardenas Cota 1996; Im et al. 1988; Morin et al. 1990; Zhang et al. 2005; Zhang et al. 2001).

These results indicate that during development of bioherbicide production protocols, factors such as efficacy and durability of the inoculum must be taken into consideration along with the yield and efficiency of fermentation.

2.4.6. Physical and environmental conditions

Environmental conditions, particularly temperature, moisture, aeration, pH and light, are important factors for growth and sporulation of microorganisms (Dhingra and Sinclair 1995). Shear (due to agitation, flask or fermentor design) and rheology are additional environmental concerns (Stowell 1991). An advantage of submerged fermentation compared to solid substrate is an enhanced ability to monitor and control these parameters (Jackson et al. 1996). There is a range of conditions for an individual microorganism that dictates the optimal, minimal, and maximal parameters for vegetative growth and sporulation. Initiation of fungal sporulation is often triggered by conditions that are adverse to vegetative growth (Dhingra and Sinclair 1995).

It is difficult to predict the performance of a complex biological system by studying the effect of only a single factor, and interactions between multiple parameters are often examined to optimize conditions (Strobel and Sullivan 1999). There can be interactions between temperature, aeration, nutrients, water potential, and other factors crucial to the growth and development of a microorganism, and the impact of these factors may sometimes need to be investigated interdependently (Stowell 1991).

Sophisticated shakers that control light, temperature, and/or agitation speed may be used for submerged fermentation of bioherbicides in shake-flasks (Auld 1992; Davis and Blevins 1979). This allows for provision of appropriate conditions to accommodate a range of requirements for different microorganisms.

2.4.6.1. Light/dark

Fungal sporulation may be stimulated by light, and this effect is closely related to nutrition and temperature (Dhingra and Sinclair 1995). De La Torre and Cárdenas-Cota (1996) outlined Kumagai's classification of three types of imperfect fungi based on light/dark cycles required for sporulation. The first type requires light for conidiophore

induction, but light also suppresses conidiation; the second type can initiate conidiophores without light, but conidia development is suppressed by blue light; and the third type is not affected by light during conidiophore induction or conidial development.

Some fungal species do not require light for growth or sporulation and can be incubated in the dark (Amsellem et al. 1999; Bolkan et al. 1982; de la Torre and Cardenas Cota 1996; Montazeri et al. 2003; Pascual et al. 1997; Winder and Van Dyke 1990; Zhang et al. 2001). Others may require light or specific cycles of light/dark (de la Torre and Cardenas Cota 1996; Figliola et al. 1988; Jamil and Nicholson 1989; Li and Holdom 1995; Montazeri and Greaves 2002; Urquhart et al. 1994; Walker and Riley 1982; Zhang et al. 2001). Even closely related fungi may have different preferences, for example, *C. gloeosporioides* f. sp. *malvae* is grown under a 12-h light cycle of fluorescent light, while *C. orbiculare* is grown in the dark (Templeton 1992).

2.4.6.2. Temperature

Optimal temperatures may be different for vegetative growth than sporulation, and often the latter has a narrower range and may require fluctuating temperatures rather than constant (Dhingra and Sinclair 1995). The preferred range for sporulation may be dictated by the environment from which the organism originated.

Liquid cultures of *C. truncatum* were incubated at 28ºC for sporulation (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002; Slininger et al. 1993). The optimal temperature for linear growth and sporulation of *C. gloeosporioides* f. sp. *aeschynomene* is 28-30°C, with no measurable growth at 16 or 40°C (Templeton 1992). Sporulation of *C. orbiculare* was found to be most profuse at 20-25°C (Templeton 1992). Temperatures from 15-27°C supported the highest yield for *C. falcatum* sporulation, and greater temperature tolerance was observed for cultures initiated from conidial suspensions than those from agar plugs of mycelium (Olufolaji 1994). Liquid cultures of *C. coccodes* were incubated at 23 or 24ºC (Yu et al. 1997; Yu et al. 1998).

Stress caused by high temperatures (37ºC) induced *P. fumosoroseus* to sporulate (de la Torre and Cardenas Cota 1996), whereas usual liquid culturing was at 28°C

(Cliquet and Jackson 2005; Jackson et al. 2004; Vega et al. 2003; Vidal et al. 1998). Other fungal biopesticide agents have showed a range of temperature requirements for optimal growth and sporulation (Amsellem et al. 1999; Bolkan et al. 1982; Cunningham et al. 1990; Engelkes et al. 1997; Figliola et al. 1988; Im et al. 1988; Li and Holdom 1995; Montazeri and Greaves 2002; Ogel et al. 1994; Ooijkaas et al. 1998; Pascual et al. 1997; Patino-Vera et al. 2005; Thomas et al. 1987; Urquhart et al. 1994; Vega et al. 2003; Walker and Riley 1982; Winder and Van Dyke 1990; Zhang et al. 2001).

2.4.6.3. Medium pH

This parameter is often overlooked during media design (Dahod 1999) but control of pH may be crucial for optimal productivity (Stanbury et al. 1995a). Media often need to be maintained at a steady pH during fermentation for optimal results but changes can be brought on by metabolism of the microorganism (eg. metabolism of nitrogen-containing salts) (Dahod 1999; Davis and Blevins 1979). Bacterial contamination of media may be reduced with a lower pH (Davis and Blevins 1979; Jackson et al. 2004).

Production of *C. truncatum* conidia was carried out at a pH of 5.0 (Bothast et al. 1993; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002; Montazeri et al. 2003; Schisler et al. 1995) or 5.5 (Jackson and Bothast 1990; Slininger et al. 1993), whereas *C. gloeosporioides* f. sp. *aeschynomene* was grown for sporulation at pH 6 (Templeton 1992). Other *Colletotrichum* spp. have been produced in liquid cultures adjusted to pH 5.0-7.0 (Templeton 1992; Yu et al. 1997; Yu et al. 1998). Studies on other fungal biopesticides have shown a range of medium pH's to be successful (Engelkes et al. 1997; Im et al. 1988; Jackson et al. 2004; Li and Holdom 1995; Montazeri and Greaves 2002; Pascual et al. 1997; Thomas et al. 1987; Urquhart et al. 1994; Van Den Boogert 1989; Walker and Riley 1982; Zhang et al. 2001).

Control of pH may be achieved with a buffer, a nutrient source, or compounds such as ammonia, hydrochloric acid, sodium hydroxide, or sulphuric acid and buffering capacity of the medium may be provided by proteins, peptides, and amino acids (Stanbury et al. 1995a).

2.4.6.4. Aeration

One of the major factors influencing a liquid culture is oxygen supplied during fermentation (Stanbury et al. 1995a). Several limiting steps can occur in aerobic shaken fermentation due to inefficient oxygen diffusion into liquid media contained in flasks (Hilton 1999). The growth rate of fungal mycelium can be limited by slow oxygen diffusion into a medium (M.D. de Jong, C.J.E.A. Bulder, C.A.G.M. Weijers, P.C. Scheepens (unpublished work, 1982)), and yield reduction and microorganism death may be a result of a lack of oxygen in aerobic fermentation (Hosobuchi and Yoshikawa 1999). The rate of oxygen transfer from gas to liquid is often the most critical parameter for aerobic fermentations and is sometimes overlooked in small fermentation systems (Hilton 1999). Oxygen availability may be influenced by the medium in terms of metabolism and rheology of certain compounds throughout the fermentation process (Stanbury et al. 1995a). The maximum rate of oxygen transfer determines the amount of cell mass that can be supported by aerobic physiology (Hilton 1999).

Normally the solubility of oxygen in water is about 6 ppm. More oxygen may be dissolved through increased aeration using agitation, which minimizes boundary layer resistance and maximizes surface area for oxygen transfer (Auld 1992; 1993). Aeration in flasks can be achieved by using variable-speed gyratory or reciprocating shakers, sparging pressurized filter-sterilized air to produce fine bubbles, or stirring with magnetic or mechanical means (Davis and Blevins 1979). Agitation on orbital shakers may be superior to reciprocating shakers to increase oxygen transfer rates, and flasks with side indentations (baffled flasks) also encourage greater turbulence and aeration (Auld 1992; 1993; Davis and Blevins 1979). However, baffled flasks may also cause splash and promote growth on flask walls, which can result in multiple simultaneous, physiologically different fermentations occurring in the same vessel (Hilton 1999). Even when this system works, it is inherently difficult to repeat or scale-up. Large-scale operations often use forced air or agitation to overcome insufficient aeration (Davis and Blevins 1979). At this scale oxygen transfer is regulated by measuring dissolved oxygen (dO) concentration and adjusting agitation speed, air-flow rate, and/or pressure (Hosobuchi and Yoshikawa 1999).

Shake cultures of *C. falcatum* at 80 RPM did not show higher sporulation when compared to stationary cultures (Olufolaji 1994). For liquid cultures of other *Colletotrichum* spp. the shaker speed was set commonly between 180 to 300 RPM (Bothast et al. 1993; Jackson and Bothast 1990; Montazeri and Greaves 2002; Yu et al. 1997).

In studying fermentation of *C. truncatum* using bench-top fermentors, Slininger *et al.,* (1993) found that the fungus required varying levels of dissolved O_2 tension (DOT) at different stages of its life cycle (i.e. germination, growth, and sporulation) for optimual yield and/or process rates. Spores produced in cultures deprived of O_2 failed to germinate, while a low level of DOT (10-20%) was sufficient to incite germination, and higher levels of DOT (30-80%) were even more favourable to spore germination. Although accumulated spores increased as the DOT increased to 55%, the yield decreased at 75% DOT due to possible O_2 toxicity. DOT equal to or greater than 55% supported high growth rates but reduced sporulation rates.

Liquid cultures of *C. gloeosporioides* f. sp. *aeschynomene* and *C. orbiculare* were aerated by continuous agitation of flask cultures or stir-tank cultures with addition of air bubbles (Templeton 1992). Desired propagules of other fungal biopesticides have been produced using a variety of shaker speeds or agitation methods to achieve required aeration levels (Amsellem et al. 1999; Cliquet and Jackson 2005; Jackson et al. 2004; Li and Holdom 1995; Ogel et al. 1994; Patino-Vera et al. 2005; Thomas et al. 1987; Urquhart et al. 1994; Vega et al. 2003; Walker and Riley 1982; Winder and Van Dyke 1990; Zhang et al. 2001).

Oxygen needs can also be influenced by the medium. For example, reduced C sources result in a higher oxygen demand (Stanbury et al. 1995a). When designing experiments and interpreting culture characteristics such as sporulation, it is important to consider O_2 transfer effects because certain conditions that increase biomass production and viscosity, such as C loading, may also increase the O_2 demand (Slininger et al. 1993). The morphological form of a fermented organism may also be affected by agitation of the culture (M.D. de Jong, C.J.E.A. Bulder, C.A.G.M. Weijers, P.C. Scheepens (unpublished work, 1982)).

2.4.7. Scale-up of fermentation

Microbial fermentation development is usually carried out in three steps: shake-flask cultures and bench-top fermentors are used initially, pilot scale for optimization of operating conditions, and plant scale for satisfaction of industrial yields and economy (Davis and Blevins 1979; Hosobuchi and Yoshikawa 1999). Physical, chemical, and process factors may need to be compensated as scale increases (Hosobuchi and Yoshikawa 1999). Scale-up is generally more practical with submerged than solid cultures (Davis and Blevins 1979).

2.4.7.1. Equipment

Small-scale fermentations, ranging from microlitre to litre volumes in flasks or carboys, have some inherent strengths and weaknesses. They are suitable for most fermentations, but are not capable of meeting all objectives and answering all questions about the requirements of the organism. There is a potential to run a large number of parallel fermentations simultaneously at relatively low costs, and initial scale-up may be as simple as increasing the number of vessels. However, limitations include a lack of monitoring capabilities, adaptive controls, and poor oxygen transfer (Hilton 1999).

Small-scale trials are usually performed in Erlenmeyer flasks or a variation of this system such as baffled flasks (Hilton 1999) for early evaluation for mass production of bioherbicides (Boyette et al. 1991). Magnetic stir cultures (M.D. de Jong, C.J.E.A. Bulder, C.A.G.M. Weijers, P.C. Scheepens (unpublished work, 1982)), and other vessels ranging from smaller-volume microplates and test tubes to larger-volume carboys can also be used at this stage (Hilton 1999); however, for the purpose of aerobic biomass production, shake-flasks remain the most popular due to greater oxygen transfer rates. Shakers can secure small fermentation vessels and agitate the culture at a set speed in an orbital or reciprocal motion and are available in a range of capacities, with or without environmental controls (Davis and Blevins 1979; Hilton 1999).

Once a flask production system has been developed, scale-up to fermentors is possible with likely minor modifications (Stowell 1991) as long as most potential problems can be resolved at the small-scale level (Davis and Blevins 1979). To produce larger quantities of bioherbicide inoculum for more elaborate greenhouse and field

studies and to fulfill commercialization potential, more precise control of growth parameters is required and a scale up to laboratory-model fermentors is essential (M.D. de Jong, C.J.E.A. Bulder, C.A.G.M. Weijers, P.C. Scheepens (unpublished work, 1982)). Laboratory models are capable of monitoring and controlling various environmental factors that may affect growth and sporulation of an organism. Key environmental factors must be monitored as closely as possible to maintain feedback control as scale increases (Hosobuchi and Yoshikawa 1999). These parameters may include temperature, agitation speed, dO, and pH (Auld 1992; Boyette et al. 1991; Davis and Blevins 1979). Additional information is accessible as scale increases, and as a result they are more clearly understood and easier to control and are inevitably more reproducible than other systems (Davis and Blevins 1979). As the scale increases, agitation power per unit volume, dO or oxygen transfer coefficient (k_La), mixing time, and impeller tip speed must be maintained (Hosobuchi and Yoshikawa 1999). Oxygen supply is optimal in fermentors because sterile air can be introduced under pressure (M.D. de Jong, C.J.E.A. Bulder, C.A.G.M. Weijers, P.C. Scheepens (unpublished work, 1982)). In large fermentors filamentous microorganisms risk shear stress, which is proportional to the tip speed of the agitation propeller and can be compensated for by using multiple or larger diameter impellors (Hosobuchi and Yoshikawa 1999).

2.4.7.2. Media considerations

Once a defined medium has been optimized, a production medium may be formulated by replacing nutritional components with complex substrates that incur a lower cost of production while retaining the quality and quantity of the final product (Boyette et al. 1991; Jackson et al. 1996; Yu et al. 1997). While exact nutrient compositions are determined at the smaller scale, minor changes may be required as scale increases (Davis and Blevins 1979). When medium composition or grades are changed or lot numbers are different from similar sources, purity or effectiveness of the final product may need to be assessed using small-scale studies (Hosobuchi and Yoshikawa 1999). The same nutrient-balanced medium can be used from small-scale to stirred and fed fermentors, but the strength of the medium may need to be changed by diluting or concentrating certain ingredients to compensate for the differing oxygen

transfer capabilities among the different sizes (Hilton 1999). The efficiency of nutrient utilization may change when fermentation is scaled up from shake-flasks to fermentors in which agitation and aeration conditions are more intense, and nutrient requirements may increase (Dahod 1999). When nutrient requirements differ between scales, nutrient concentration changes may be met through feeding (Hosobuchi and Yoshikawa 1999). As scale increases, certain ingredients may be replaced with nutritionally suitable low-cost complex substrates or some readily utilizable sources that would otherwise likely affect the pH characteristics if used on a smaller scale lacking suitable pH control (Dahod 1999; Jackson 1997)

2.4.7.3. Inoculum preparation

Inoculum considerations for scale-up fermentations of sporulating organisms include the following (Stanbury et al. 1995b): a master culture reconstituted and sub-cultured; shake-flasks are inoculated as viability checks, then used as seed inoculum; these cultures are used to inoculate either larger flasks or laboratory fermentors and the process is repeated with progressively larger vessels until the desired scale is reached. Contamination checks must be performed at each stage to ensure purity of cultures (Davis and Blevins 1979; Stanbury et al. 1995b).

Smaller fermentors (less than 4L) are usually used for seed inoculum, while scale-up studies are then carried out in fermentors of 4-28 L, followed by intermediate increases using 30-150 L or larger fermentors (Davis and Blevins 1979). For best results, inoculum should always be in an appropriate physiological condition before being transferred to the next stage. Growth rate, indicated by oxygen uptake or carbon dioxide evolution rates, may be used to determine the timing for transfer of inoculum into the next level (Monaghan et al. 1999).

Steps of pre- or seed cultures increase as the size of the production increases (Hosobuchi and Yoshikawa 1999), but the number of sub-cultures may affect productivity and stability of an organism (Monaghan et al. 1999), such as virulence of a fungal pathogen. Therefore the preservation of efficacy of inoculum produced at different scales must be confirmed.

2.4.8. Downstream processing

Downstream processing entails harvesting of the desired products from the point the culture leaves the fermentation vessel. These processes include concentration, stabilization, and drying/packaging of materials if applicable (Stowell 1991). Challenges may occur at any step as production scale increases, and specialized equipment is often required (Davis and Blevins 1979).

It is important that fermentation media will not hinder the downstream processing steps (Dahod 1999). Homogeneity of the medium facilitates processing of liquid cultures (Jackson et al. 1996). Product recovery and effluent treatment can be more problematic when undefined media are used because some components of the complex nutrient source may not be consumed (Stanbury et al. 1995a). Biomass is usually harvested by centrifugation or filtering processes (Davis and Blevins 1979). Recovery of spores from submerged cultures of filamentous fungi can be a challenge, as a large number of spores may be caught in mycelial biomass and specialized centrifuges may be required to separate them (Auld 1992; 1993). Another benefit to optimizing production of desired propagules is that sometimes when spore yields are high, there may be fewer mycelial fragments present in the cultures (Thomas et al. 1987). Conidia of *C. gloeosporioides* f. sp. *aeschynomene* and *C. orbiculare* are harvested by filtering out mycelia from the culture suspension with cheesecloth, and concentrating by centrifugation (Templeton 1992). The efficiency of downstream processing starts with medium design and should be optimized each step of the way during production.

3. MATERIALS AND METHODS

3.1. Preparation of fungal stock culture

Colletotrichum truncatum isolate 00-3B1, originally obtained from a diseased scentless chamomile plant near Hafford, Saskatchewan and preserved at -80ºC, was applied to scentless chamomile plants in a research greenhouse to confirm pathogenicity. To reisolate the fungus, pieces (0.5 cm) of stem and leaf materials from lesion edges were removed and surface sterilized in 70% v/v ethanol (10 s) followed by 0.6% v/v sodium hypochlorite (1 min), then rinsed in sterilized distilled water, blotted dry on sterile paper towels, and placed on potato dextrose agar (PDA, Difco Laboratories, St. Louis, MO) amended with penicillin and streptomycin (100 ppm) in 8.5 cm Petri plates. Plates were placed in an incubator (Conviron Inc., Winnipeg, MB) at an ambient temperature of 20°C with a 14-h fluorescent photoperiod (28 μmol m^{-2} s^{-1}) for 7 d. To obtain a pure culture, mycelium pieces were removed from edges of fungal colonies and transferred to V8® juice (V8) agar (200 ml V8® juice, 3 g $CaCO_2$, 20 g agar, 800 ml distilled water) in 8.5 cm Petri plates. Plates were then incubated under the same conditions for 14 d to allow conidiation (sporulation) of the fungus.

Sporulating plates were flooded with sterile water and scraped with a sterilized glass 'hockey stick' to dislodge conidia (spores). The resulting suspension was centrifuged and conidial pellets were resuspended with water or liquid media to alleviate potential impact by a hydrophilic mucilaginous spore matrix found commonly with colletotrichum fungi that may reduce spore germination (Bailey et al. 1992; Cunningham et al. 1990; Yu et al. 1998). The spore concentration was estimated with the aid of a haemocytometer (Hausser Scientific, Horsham, PA). Cultural purity was determined by streaking the suspension on V8 and nutrient agar (NA, Sigma-Aldrich, St. Louis, MO) and incubating at 30°C for 1 wk for visualization of potential fungal and bacterial contaminants. Any culture with incidence of contamination was discarded or purified by transferring onto antibiotic agar or repeating the inoculation and reisolation steps described above.

A working cell bank was created by preparing a stock of liquid culture inoculum for storage at -80°C. First 50 µl of a spore (sp) suspension (1 x 10^7 sp ml^{-1}) was added to 50 ml of nutrient broth (NB, Sigma-Aldrich) containing 100 ppm of antibiotic (penicillin plus streptomycin, chloramphenicol, or tetracycline) in multiple 250-ml Erlenmeyer flasks. These were incubated on an environmentally controlled shaker at 22°C and 150 rpm (Innova 4230, New Brunswick Scientific Co., Edison, NJ) for 1 wk. Then 1 ml of one of these cultures was transferred to 100 ml of a defined basal salts medium (DBSM, Table 3.1) and incubated initially at 20°C for 1 wk followed by another wk at 12°C. Spores were harvested through centrifugation, resuspended to approximately 1 x 10^7 sp/ml in a cryofreezer preservation solution (10 g skim milk powder, 40 ml glycerol, 160 ml sterilized water), and stored in cryovials (Fisher Scientific, Edmonton, AB) in an ultra-low freezer (Model 982, Forma Scientific, Inc., Marietta, OH) at -80°C. The preservation solution can help protect fungal spores in deep freezing conditions (Monaghan et al. 1999). Viability and purity of stock cultures were confirmed by culturing on NA and V8 agar at 30°C for 1 wk.

3.2. Preparation of liquid media

Media were prepared by mixing ingredients in water on a stir plate. A defined basal salts medium (DBSM, Table 3.1), adopted from Jackson et al. (1996), was used for liquid culture experiments. The pH of liquid media was measured with a Cardy Twin pH Meter (Spectrum Technologies, Inc., Plainfield, IL), and unless otherwise stated, was adjusted to 5.5 with 1N HCl or 1N NaOH (Jackson et al. 2004; Jackson and Slininger 1993; Montazeri et al. 2003). For all shake-flask experiments, 100 ml of media were added to 500-ml sized flasks. Three or four replicate regular Erlenmeyer flasks were used for each treatment in all experiments, while aeration experiments also included baffled flasks. Fungal inoculum used for medium inoculation in bench-top fermentation was produced in 1000-ml baffled flasks containing 200 ml of media. Flask openings were plugged with plastic foam stoppers and wrapped with aluminum foil to create a sterile, selectively permeable barrier that allows gas exchange (Hilton 1999). Flasks containing media were autoclaved for 20 min at 121°C, 15 PSI and were kept at 20°C for at least 24 h before inoculation, in order to allow visual inspection for contamination.

Table 3.1. Composition of the defined basal salts medium

Ingredient	Amount (/L)	Supplier
Distilled Water	1000 ml	
KH_2PO_4	2.0 g	J.T. Baker
$CaCl_2 \cdot 2H_2O$	0.4 g	Fisher Scientific
$MgSO_4 \cdot 7H_2O$	0.3 g	Fisher Scientific
Stock Solution A[a]:		
$CoCl_2 \cdot 6H_2O$	37.0 mg	J.T. Baker
$FeSO_4 \cdot 7H_2O$	50.0 mg	J.T. Baker
$MnSO_4 \cdot H_2O$	16.0 mg	BDH Chemicals
$ZnSO_4 \cdot 7H_2O$	14.0 mg	J.T. Baker
Stock Solution B[b]:	500 µg each	
Thiamine		Sigma-Aldrich
Riboflavin		Sigma-Aldrich
Pantothenate		Calbiochem
Niacin		Calbiochem
Pyridoxamine		Sigma-Aldrich
Thiotic acid		Sigma-Aldrich
Stock Solution C[b]:	50 µg each	
Folic acid		Sigma-Aldrich
Biotin		Calbiochem
Vitamin B_{12}		Sigma-Aldrich
C source	9.6 g glucose	Fisher Scientific
N source	2.45 g casamino acids (unless otherwise stated)	Fisher Scientific

[a] autoclaved separately
[b] filter sterilized, added to media after autoclaving

3.3. Liquid culturing and fungal inoculum harvesting

The liquid medium was inoculated with a spore suspension obtained from working cell banks, or from sporulating V8 agar or shake-flask liquid cultures. Unless otherwise stated, 100 µl of this suspension (1×10^7 sp/ml) was used to inoculate each 100 ml of media. Flasks were incubated for 14 days in incubator shakers (New Brunswick Scientific Co.) or on an industrial shaker in a temperature-controlled room. Optimized parameters from flask fermentations were further examined for scale-up in 20-L fermentors (Biostat C-20, B. Braun Biotech, Goettingen, Germany) with a DCU3 controller. The inoculum used for each 20-L fermentor was 200 ml of 7.5×10^4 sp/ml

plus mycelium. Fermentor cultures were grown at 20°C for 1 wk followed by 12°C for 1 wk.

Unless otherwise stated, shaker speed for all experiments was 150 RPM, and flasks were incubated without supplemented lighting. For experiments on effects of initial inoculum concentration, C and N sources, C concentration, C:N ratio, light, and pH, flask cultures were incubated at an ambient temperature of 16°C. After an optimal temperature regime was determined, experiments on effects of aeration or carbon-aeration interaction, and scale-up, were incubated at 20ºC for 1 wk followed by 12ºC for 1 wk. Every 2-4 d, cultures were gently swirled by hand until all accumulated growth on the sides of the flasks was distributed back into the medium to avoid build-up of mycelial growth on flask walls (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002). If this routine was not maintained, a ring of mycelia growth often formed and fell into the medium, and coalesced or formed clumps, resulting in poor homogeneity of the culture and reduced sporulation.

Glucose concentration in liquid cultures was monitored in several trials as an indicator of C consumption during fermentation using the YSI Select Biochemistry Analyser (Model YSI 2700, YSI Inc., Yellow Springs, OH) located at the Saskatchewan Research Council (SRC). The instrument was set for D-glucose/dextrose chemistry performance specifications with YSI membrane 2365. Cultural samples of 2-5 ml were passed through a 0.45 µl filter to remove fungal fragments prior to the analysis. Each sample was tested twice and readings averaged.

Culture samples were collected during and at the end of fermentation for assessment of sporulation, and biomass where applicable. Sampling frequency was reduced for small-volume experiments to minimize contamination or interruption of cultural/environmental conditions (Hilton 1999). Prior to sampling, flask cultures were swirled for about 10 s to ensure homogeneity and 1 ml for spore yield determination was pipetted to a micro-centrifuge tube for storage at 4°C until analysis was performed.

To determine the spore yield, sample tubes were vortexed for about 10 s and the spore concentration was estimated with aid of a haemocytometer. For concentrations below 1.5 x 10^6 sp/ml, all spores in two sets of 25 haemocytometer grids were counted

(n spores counted = n x 10^4 sp/ml). For concentrations over 1.5 x 10^6 sp/ml, only spores present in 5 of the squares were counted (n spores counted = n x 5 x 10^4 sp/ml). Samples above 1.5 x 10^7 sp/ml were diluted to between 1.5 x 10^5 and 1.5 x 10^7 with water before counting. The spore yield (sp/ml) was then obtained by taking the average of two separate counts from both counting areas of the haemocytometer for each sample. Specific spore yield (SSY, sp/g glucose), which was based on the conversion of glucose to spores, was calculated using the following formula (Yu et al. 1998):

SSY = sp yield per L culture / g initial glucose per L medium

To measure total fungal biomass, either entire flask contents containing mycelium were collected after spore harvest, or a 30-ml homogenous culture sample was taken (including mycelium, spores and microsclerotia), centrifuged at 8500 rpm for 10 min, media were decanted, and spores were resuspended in 30 ml of water before being placed in pre-weighed aluminium trays and dried at 60°C (Fisher Isotemp ® Oven, Edmonton, AB) for 48 h before dry weight was taken. Dry weight values were converted to g/L of culture prior to statistical analysis. Specific biomass yield (SBY, g biomass/g glucose), which was based on the conversion of glucose to biomass, was calculated using the following formula:

SBY = g biomass yield per L culture / g initial glucose per L medium

Spores were harvested by first straining flask contents through two layers of cheesecloth to separate spores from mycelium. This spore suspension was centrifuged (IEC Centra MP4, Thermo Electron Company, Waltham, MA) at 8500 rpm for 10 min, which usually precipitated peach-coloured spores along with black-coloured microsclerotia. Centrifugation is frequently used for harvesting fungal propagules produced with liquid fermentation (Auld 1993; Hilton 1999) and the method used in this study was similar to those used for collecting spores from liquid cultures of other colletotrichum bioherbicide agents including BioMal® and Collego® (Cunningham et al. 1990; Templeton 1992). Pellets of spores were resuspended in 0.1% v/v Tween® 80 (surfactant, Fisher Scientific) for plant inoculation. Following large-scale fermentation, contents of the 20-L fermentors were drained into Nalgene® wide-mouth carboys and passed through layers of cheesecloth into additional carboys. The mycelium-free

suspension was centrifuged in a basket centrifuge (BEP 305TX, Bock Engineered Products, Inc, Toledo, OH) to collect spores, which were then stored at 4°C.

3.4. Plant preparation and inoculation

Scentless chamomile seeds were harvested in 2001 from mature plants located in the Hafford area of Saskatchewan and stored at room temperature. To produce transplants, seeds were sown to a soil-less mix consisting of one part sand to 12 parts of a 1:2 sphagnum peat moss-vermiculite mix plus 1% (wt/v) 16:8:12 N-P-K fertilizer and covered with an 1-cm layer of Redi-earth. After 10-14 d in a greenhouse (20±3°C, supplementary lighting for 16 h photoperiod using high pressure sodium bulbs), seedlings at 2- to 4-leaf stages were transplanted to 7.5-cm pots filled with the soil-less mixture described above. Each pot contained 1 plant, which formed an experimental unit.

After another 10-20 d, plants at 5- to 8-leaf stages were sprayed with spore suspensions of the fungus using an airbrush sprayer (Paasche Airbrush Ltd., Chicago, IL) at 275 kPa air pressure in a spray cabinet (Haltech Ag GPS, Guelph, ON) to confirm or compare weed control efficacy of fungal spores produced under various experimental conditions. Approximately 20 ml of spore suspension (1 x 10^5 sp/ml, 0.1% Tween® 80) were applied to achieve runoff on the foliage of 4 plants (replicates per treatment). An additional 4 plants were sprayed with 20 ml of 0.1% Tween® 80 as a negative control. Unless otherwise stated, spores from each treatment of fermentation experiments were tested for efficacy against scentless chamomile.

Treated plants were placed in a dew chamber (Percival Scientific I-35 DLM, Boone, IA) for 24 h in darkness, with 100% relative humidity and an ambient temperature of 20° C (achieved with settings of ~35°C water temperature and ~5°C wall temperature). A 24-h dew period is required for maximum infection of scentless chamomile by this bioherbicide agent (Graham et al. 2005; Peng et al. 2005). After the dew period, plants were then placed in the greenhouse under the same conditions as previously described for 1 wk, at which time fresh weight of each individual plant was taken as a measure of weed control using methods reported previously (Graham 2004). Prior to statistical analyses, plant fresh weight was converted to fresh weight reduction

(FWR, %) to compensate for differences in overall plant size between experiments or trial repetitions. All control plants had 0% FWR value with no variance, and therefore were excluded from analysis of variance. The mean fresh weight of control plants in a trial was calculated (CFW) and the fresh weight of each treated plant (TFW) was used to calculate a value of percent FWR using the following formula:

$$FWR = (CFW - TFW) / CFW \times 100$$

Diseased plant materials were periodically collected and the causal agent reisolated to confirm authenticity and pathogenicity of the organism (Agrios 1997; Chandramohan and Charudattan 2001).

3.5. Experimental design and statistical analysis

Flask culture and efficacy experiments used a completely randomized design (CRD) with three or four replicate flasks or plants. Two experiments, (i.e. the effect of complex and defined liquid media on fungal sporulation at different temperatures, and effect of aeration and glucose concentration on sporulation), also used CRD but with a factorial arrangement. All experiments were repeated once, unless stated otherwise. Data for spore enumeration and weed control efficacy were subjected to Analysis of Variance (ANOVA) using the Statistical Analysis System (SAS) version 8.2 (SAS Institute Inc., Cary, NC) PROC GLM procedure. Mean separation was performed using the least significant difference (LSD) (P=0.05) if the ANOVA was significant (Fisher's protected LSD). Fisher's protected LSD has been used for mean separation in other similar fermentation experiments (Jackson and Slininger 1993).

Bartlett's test of homogeneity of variance was performed before any experiment repetition data were pooled (Snedecor and Cochran 1980). When the variances were non-homogeneous, data from repeated trials were reported separately. This method of data treatment has been used previously (Yu et al. 1997; Yu et al. 1998).

When the purpose of an experiment was to explore quantitative relationships between two factors, regression analysis was performed (Little and Hills 1978) using SigmaPlot version 9.0 (Systat Software, Inc., San Jose, CA).

3.6. Experimental treatments

3.6.1. Medium type and incubation temperature

This preliminary experiment was designed to explore the temperature range for sporulation of the fungus in complex and defined liquid media using shake-flask cultures. A defined liquid medium (DBSM: Table 3.1) was compared to a complex liquid medium (V8 medium: 200 ml V8® juice, 3 g $CaCO_2$, 800 ml distilled water). Cultures were incubated at 16, 22, and 28°C in darkness. The following treatments were included:

1. V8 medium at 16°
2. V8 medium at 22°
3. V8 medium at 28°
4. DBSM at 16°
5. DBSM at 22°
6. DBSM at 28°

3.6.2. Initial inoculum concentration

Inoculum was prepared in the same liquid medium used for fermentation in order to avoid dilution of the medium by the inoculum. The following treatments were included with the final volume of the DBSM adjusted to 100 ml for each flask to compensate for different inoculum volumes:

1. 0.01 ml of 1 x 10^7 sp/ml in 99.99 ml medium = 1 x 10^3 sp/ml
2. 0.1 ml of 1 x 10^7 sp/ml in 99.9 ml medium = 1 x 10^4 sp/ml
3. 1.0 ml of 1 x 10^7 sp/ml in 99 ml medium = 1 x 10^5 sp/ml
4. 10.0 ml of 1 x 10^7 sp/ml in 90 ml medium = 1 x 10^6 sp/ml

3.6.3. Carbon sources

Carbon content was calculated according to % of total molecular weight, and was added to the DBSM at a concentration of 10.3 g of C/L. Casamino acids were used as an N source and C:N ratio was kept the same for all treatments at 23:1. The following treatments were included:

1. trehalose ($C_{12}H_{22}O_{11}\cdot 2H_2O$)
2. fructose ($C_6H_{12}O_6$)
3. sucrose ($C_{12}H_{22}O_{11}$)
4. lactose ($C_{12}H_{22}O_{11}$)
5. maltose ($C_{12}H_{22}O_{11}$)
6. cellulose ($C_6H_{12}O_6$)
7. glycerol ($C_3H_8O_3$)
8. molasses (approximately 70% C)
9. glucose ($C_6H_{12}O_6$)

3.6.4. Nitrogen sources

Nitrogen content was calculated according to % of total molecular weight or the manufacturer's analysis. For casamino acids and casein enzymatic hydrolysate, analysis was performed by Dr. Renato de Freitas (College of Agriculture, U of S) to confirm manufacturer's analysis. Glucose was used to supplement C required to maintain a concentration of 10.3 g/L in the DBSM, with a C:N ratio of 23:1. The following treatments were included:

1. casamino acids (31.8%C, 9.36% N)
2. L-leucine ($C_6H_{13}NO_2$)
3. L-glutamic acid ($C_5H_9NO_4$)
4. ammonium nitrate ($NH_4\ NO_3$)
5. potassium nitrate (KNO_3)
6. casein enzymatic hydrolysate (45.7% C, 13.5% N)
7. cottonseed hydrolysate (9.472% N)
8. tryptone (13.3% N)

3.6.5. Glucose concentration

Nine glucose concentrations were used, ranging from 0 to 40 g/L glucose in the DBSM (equivalent to 0 to 20.6 g/L C), with casamino acids as a N source and the C:N ratio held constant at 23:1 for all treatments. The following treatments were included:

1. 40g/L glucose
2. 35 g/L glucose
3. 30 g/L glucose
4. 25 g/L glucose
5. 20 g/L glucose
6. 15 g/L glucose
7. 10 g/L glucose
8. 5 g/L glucose
9. 0 g/L glucose

3.6.6. Carbon-to-nitrogen ratio

Glucose and casamino acids were selected as C and N sources in the DBSM, with C concentration held constant at 10g/L glucose (about 5.15 g/L total C) for all treatments. The following treatments were included:

1. 5:1 ratio
2. 7:1 ratio
3. 10:1 ratio
4. 15:1 ratio
5. 20:1 ratio
6. 23:1 ratio
7. 25:1 ratio
8. 30:1 ratio
9. 40:1 ratio

3.6.7. Light/dark conditions

For investigation of the effect of light/dark conditions on sporulation, two Innova 4230 Refrigerated Incubator Shakers (New Brunswick Scientific Co.) were used with the interior lights left on at all times (13 watt – TL13/35 – vertical fluorescent light). Flasks were covered with tinfoil for the appropriate time durations to create darkness. The following treatments were included:

1. 24 h darkness
2. 14 h light/10 h darkness
3. 24 h light

3.6.8. Fermentation temperature

Two Innova 4230 Refrigerated Incubator Shakers (New Brunswick Scientific Co.) were set at 12° and 20° in addition to a G-53 Industrial Shaker (New Brunswick Scientific Co.) which was maintained in a temperature controlled room at 16°C. The following treatments were included:

1. 12°C constant for 2 wk
2. 12°C 1 wk, then 16°C 1 wk
3. 12°C 1 wk, then 20°C 1 wk
4. 16°C constant for 2 wk
5. 16°C 1 wk, then 12°C 1 wk
6. 16°C 1 wk, then 20°C 1 wk
7. 20°C constant for 2 wk
8. 20°C 1 wk, then 12°C 1 wk
9. 20°C 1 wk, then 16°C 1 wk

3.6.9. Medium pH

Various medium pH levels were obtained by adding 1 N NaOH or 1 N HCl. Due to the potential impact of autoclaving on pH levels (Dhingra and Sinclair 1995), two separate experiments were conducted with the pH being adjusted prior to and after autoclaving. The pH was monitored but not adjusted during culturing. The following initial pH levels were investigated:

1. pH 4.5
2. pH 5.5
3. pH 6.5
4. pH 7.5

3.6.10. Aeration

Varying levels of aeration were created by using two types of flask designs (standard Erlenmeyer flasks and baffled flasks) as well as different shaker speeds (50 - 200 RPM). Gas-liquid mass transfer (K_La) can be increased by adjusting parameters that affect the gas-liquid mass transfer coefficient (K_L) or the gas-liquid interfacial area (a). The latter can be increased by decreasing the liquid volume relative to the vessel volume, increasing shaker speed or diameter, or modifying the vessel with baffles to increase turbulent flow (Hilton 1999). In this experiment, higher aeration, simulating higher dissolved oxygen (dO), was created by using baffled flasks and faster shaker speeds. A shaker speed of 300 RPM was also attempted, but this resulted in splashing and more growth on the sides of the flasks than in the liquid without noticeable sporulation, and this treatment was abandoned. The following treatments were included:

1. 50 RPM – standard flask
2. 100 RPM – standard flask
3. 150 RPM – standard flask
4. 200 RPM – standard flask
5. 50 RPM – baffled flask
6. 100 RPM – baffled flask
7. 150 RPM – baffled flask
8. 200 RPM – baffled flask

3.6.11. Interaction between aeration and glucose concentration

Glucose and casamino acids were used as C and N sources in the DBSM. Four glucose concentrations were used, ranging from 5g to 40g/L (approximately 2.6 g to 20.6 g/L total C content), with C:N ratio held constant at 23:1 by adjusting the amount of casamino acids accordingly at each glucose concentration. Low and high aeration levels were established using regular Erlenmeyer flasks at 100 RPM shaker speed and baffled flasks at 200 RPM, respectively. The following treatments were considered:

1. 40 g/L glucose — low aeration
2. 20 g/L glucose — low aeration
3. 10 g/L glucose — low aeration
4. 5 g/L glucose — low aeration
5. 40 g/L glucose — high aeration
6. 20 g/L glucose — high aeration
7. 10 g/L glucose — high aeration
8. 5 g/L glucose — high aeration

3.6.12. Scale-up of production using 20-litre fermentors

Optimized parameters from flask fermentations were further examined for scale-up in 20-L fermentors (B. Braun Biotech) with a DCU3 controller, supplied with 60%, 30%, and 10% dissolved oxygen (dO). Levels of dO at 10%, 30%, and 60% were maintained with a dO cascade of: agitation 60-165 RPM, back pressure 0.2-0.5 bar, and airflow 0.1-0.5 vvm (volume of air per fermentor volume per minute). The initial glucose concentration was 5.5 g/L with a 23:1 C:N ratio. The temperature regime was 20ºC for one week followed by 12ºC for one week. The pH was adjusted to 7.5 at the start of the run and was monitored but not adjusted after this point. Glucose concentration (C exhaustion) was monitored every 24 h, whereas fungal biomass and sporulation were measured every 48 h.

4. RESULTS

4.1. Medium type and incubation temperature

Spore yield was measured at 7, 10, and 14 d. There was no significant effect of medium type or temperature on sporulation after 7 d (ANOVA, P>0.05), while after 10 and 14 d there was significant interaction between medium type and incubation temperature (ANOVA, P<0.05), and the main effect was also significant for both factors.

At 14 d the fungus produced significantly more spores in DBSM than in the V8 medium and significantly fewer spores at 28ºC than at lower temperatures. Overall, sporulation was significantly better at 16-22ºC in DBSM compared to other treatments (Table 4.1).

Biomass was collected from all of the flasks from one trial at 14 days. Mycelium in the V8 was in the form of a thick, dispersed mass, while more distinctive mycelial balls were formed in the DBSM. The fungus produced significantly higher biomass in DBSM than in V8 and significantly less biomass at 28ºC than at lower temperatures. Overall, biomass was significantly higher at 16-22ºC in DBSM and lower at 28ºC in V8 when compared to other treatments (Table 4.2).

Table 4.1. Mean spore yield in DBSM and V8 at different temperatures after 14 d

Spore yield (sp/ml)[a]							
Medium type	Temperature						Spore yield (sp/ml)[c]
	16º		22º		28º		
DBSM	6.13×10^6	a	5.23×10^6	a	1.18×10^5	b	3.83×10^6 a
V8	3.07×10^5	b	1.23×10^4	b	7.41×10^2	b	1.07×10^5 b
Spore yield (sp/ml)[b]	3.22×10^6	a	2.62×10^6	a	5.95×10^4	b	

[a] Means of 6 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, *P*=0.05).

[b] Temperature treatment means of 12 replicates.

[c] Medium type treatment means of 18 replicates.

Table 4.2. Mean biomass in DBSM and V8 at different temperatures

Biomass (g/L)[a]								
Medium type	Temperature							
	16º		22º		28º		Biomass (g/L)[c]	
DBSM	7.25	a	7.71	a	4.02	b	6.33	a
V8	4.11	b	3.54	b	2.00	c	3.22	b
Biomass (g/L)[b]	5.68	a	5.63	a	3.01	b		

[a] Means of 3 replicates from 1 trial. Means with the same letter(s) are not significantly different (LSD, *P*=0.05).

[b] Temperature treatment means of 6 replicates.

[c] Medium type treatment means of 9 replicates.

4.2. Initial inoculum concentration

There was a trend that lower initial inoculum concentrations favoured higher sporulation of the fungus (Figure 4.1), indicated by linear regression with a significant correlation coefficient. In the first trial, the effect was not significant (ANOVA, P>0.05), while in the second trial initial inoculum concentrations at 10^3 and 10^4 sp/ml yielded significantly higher sporulation than did 10^5 or 10^6 sp/ml (Table A.1, Appendix).

The effect of initial inoculum concentration on biomass was significant (ANOVA, P<0.05) in combined trials, with initial inoculum concentrations at 10^3 and 10^5 sp/ml yielding significantly higher than 10^4 sp/ml (Table 4.3).

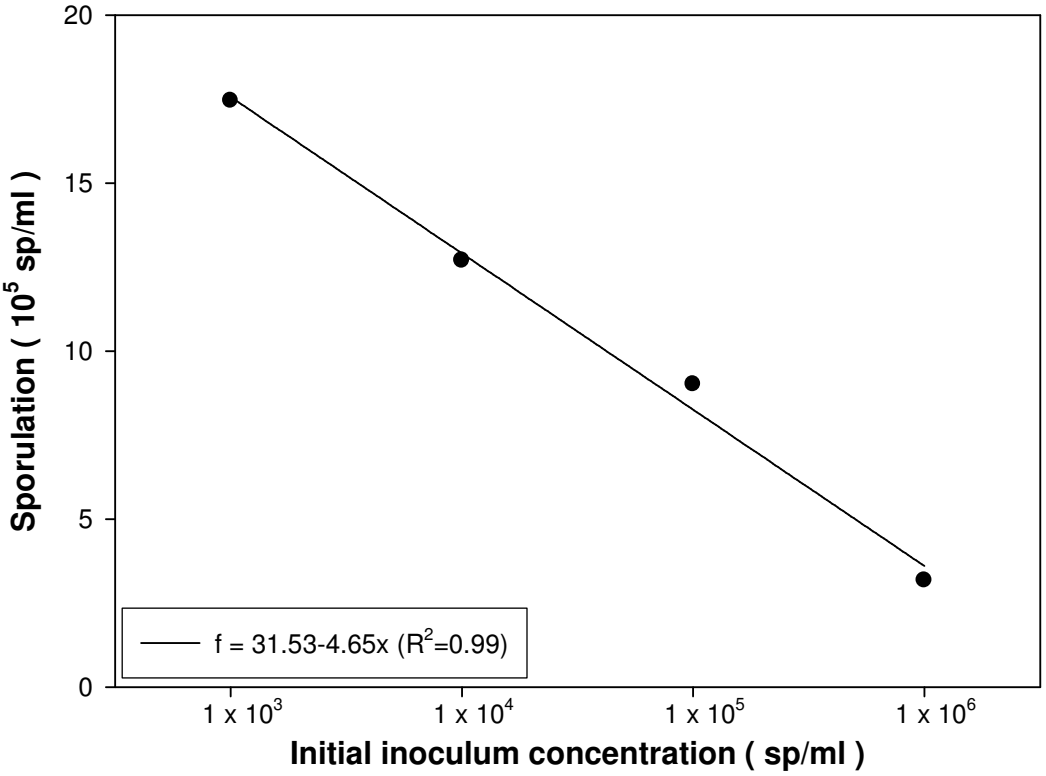

Figure 4.1. Effect of initial inoculum concentration on spore yield (initial inoculum concentration logarithmic scale). Linear regression analysis of means of 8 replicates from 2 trials.

Table 4.3. Effect of initial inoculum concentration on biomass production

Initial inoculum concentration (sp/ml)	Biomass (g/L)[a]	
1×10^3	3.66	a
1×10^4	2.66	b
1×10^5	3.69	a
1×10^6	3.01	ab

[a] Means of 6 replicates from 2 trials. Means with the same letter(s) are not significantly different (LSD, P=0.05).

4.3. Carbon sources

Spore yields were significantly higher when the DBSM was amended with sucrose or glucose compared to lactose, cellulose, fructose, or molasses, while biomass was significantly higher with cellulose than all other C sources tested (Table 4.4).

Enough spores were harvested from five of the treatments in the first trial to meet the minimum dose requirement for plant inoculation. All treatments caused infection on scentless chamomile plants, regardless of the carbon source used in the inoculum production medium, with significantly lower fresh weights when compared to the control (Table A.2, Appendix). Inoculum produced in media amended with glucose or fructose caused significantly greater fresh weight reduction than maltose (Table 4.5).

Table 4.4. Effect of C source on spore yield and biomass production

Carbon source	Spore yield (sp/ml)[a]		Biomass (g/L)[b]	
Sucrose	1.19×10^6	a	5.23	b
Glucose	1.03×10^6	ab	3.88	bcd
Trehalose	3.19×10^5	abc	1.65	e
Maltose	1.64×10^5	bc	0.86	e
Glycerol	1.16×10^5	bc	1.99	de
Lactose	6.34×10^4	c	0.88	e
Cellulose	5.58×10^4	c	8.11	a
Fructose	1.45×10^4	c	2.61	cde
Molasses	0	c	4.49	bc

[a] Means of 6 replicates from 2 trials. Spore yields with the same letter(s) are not significantly different (LSD, P=0.05).

[b] Means of 6 replicates from 2 trials. Biomass means with the same letter(s) are not significantly different (LSD, P=0.05).

Table 4.5. Effect of C source used in the inoculum production medium on efficacy of *C. truncatum* against scentless chamomile

Carbon source	Fresh weight reduction (%)[a]	
Sucrose	57.1	ab
Glucose	64.6	a
Trehalose	56.8	ab
Maltose	43.2	b
Fructose	70.9	a

[a] Means of 4 replicates from one trial. Means with the same letter(s) are not significantly different (LSD, P=0.05).

4.4. Nitrogen sources

When the DBSM was amended with casamino acids, cottonseed hydrolysate or casein enzymatic hydrolysate there were similar spore numbers, while casein enzymatic hydrolysate yielded significantly more spores than treatments supplemented with other N sources including L-leucine, L-glutamic acid, ammonium nitrate, potassium nitrate, and tryptone (Table 4.6).

Enough spores were harvested from two of the treatments of the first trial to meet the minimum dose requirement for plant inoculation. Both treatments caused infection on scentless chamomile plants, with significantly lower fresh weights when compared to the control (Table A.3, Appendix), but overall the efficacy of inoculum in this trial was poor. Fresh weight reduction caused by inoculum produced in media amended with casein enzymatic hydrolysate was not significantly different than casamino acids (Table 4.7).

Table 4.6. Effect of N source on spore yield

Nitrogen source	Spore yield (sp/ml)[a]	
Casein enzymatic hydrolysate	1.03×10^6	a
Cottonseed hydrolysate	7.58×10^5	ab
Casamino acids	2.21×10^5	ab
Ammonium nitrate	1.62×10^5	b
Tryptone	1.58×10^5	b
L-leucine	1.13×10^5	b
Potassium nitrate	6.61×10^4	b
L-glutamic acid	4.36×10^4	b

[a] Means of 6 replicates from 2 trials. Means with the same letter(s) are not significantly different (LSD, P=0.05).

Table 4.7. Effect of N source used in the inoculum production medium on efficacy of *C. truncatum* against scentless chamomile

Nitrogen source	Fresh weight reduction mean (%)[a]	
Casein enzymatic hydrolysate	34.0	a
Casamino acids	16.9	a

[a] Means of 6 replicates from one trial. Means with the same letter(s) are not significantly different (LSD, P=0.05).

4.5. Glucose concentration

As the glucose concentration in the medium increased from 0 to 40 g/L, biomass and sporulation also increased. This was illustrated using curvilinear lines for both biomass and sporulation (Figure 4.2). When the medium was amended with 35-40 g/L glucose, there were significantly more spores and biomass produced than in media with lower glucose concentrations (Table A.4, Appendix).

There was no significant difference in specific spore yield from 5-40 g/L, with the exception of 20 g/L, which was significantly lower than 35-40 g/L (Table A.5, Appendix). There was no significant difference in specific biomass yield from 5-40 g/L, with the exception of 15 g/L, which was significantly lower than 5 g/L (Table A.5, Appendix).

All bioherbicide treatments caused infection on scentless chamomile plants regardless of the glucose concentration in the medium used to produce the inoculum, with significantly lower fresh weights when compared to the untreated control (Table A.6, Appendix). The trend indicates that efficacy increased (higher fresh weight reduction) as the glucose concentration in the medium used to produce the inoculum decreased (Figure 4.3 and Figure 4.4), with fresh weight reduction caused by inoculum produced at 5-10 g/L significantly greater than inoculum produced at 40 g/L (Table A.7, Appendix).

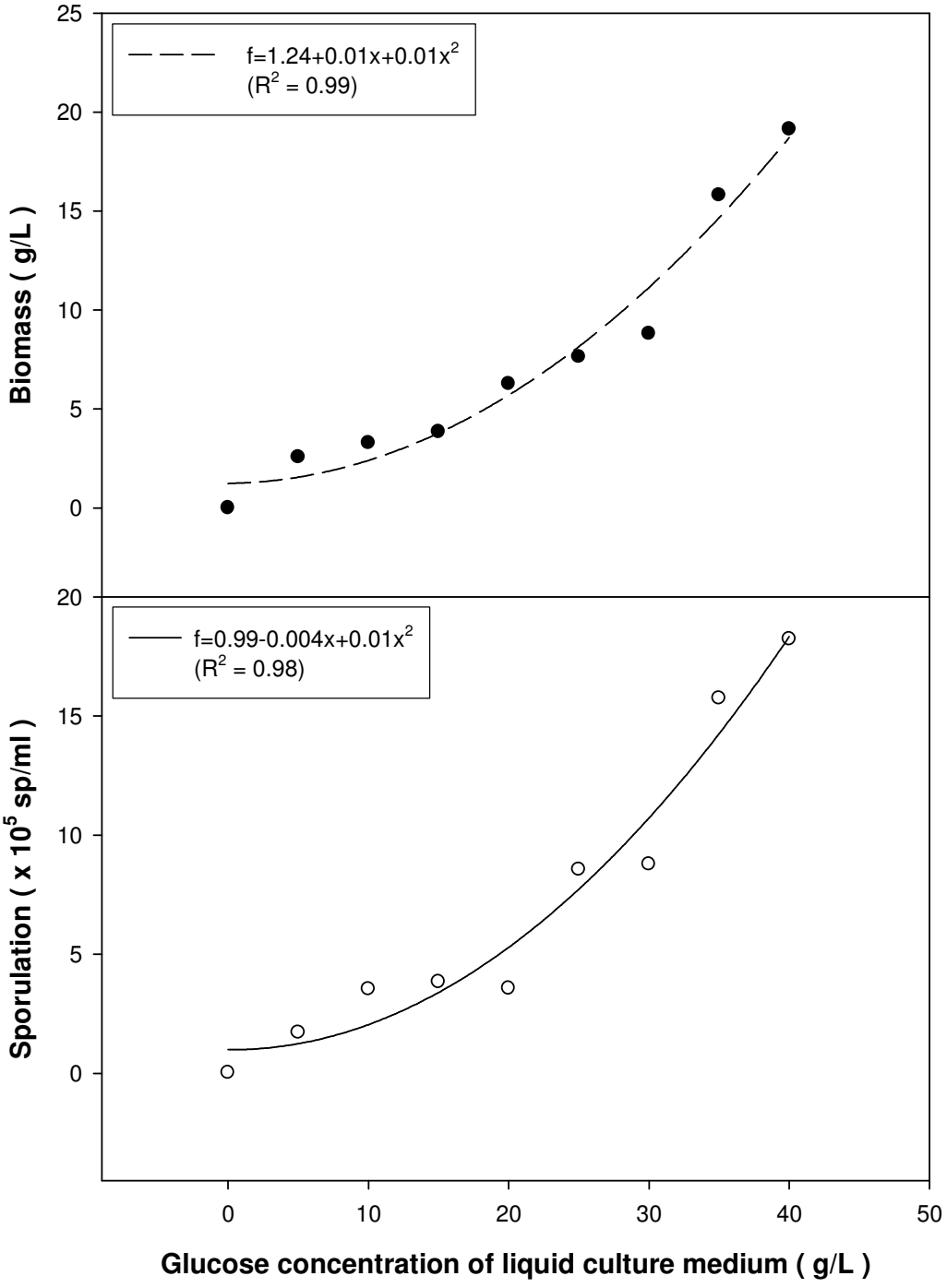

Figure 4.2. Effect of glucose concentration in the medium on biomass production and spore yield. Quadratic regression analysis of means of 8 replicates from 2 combined trials.

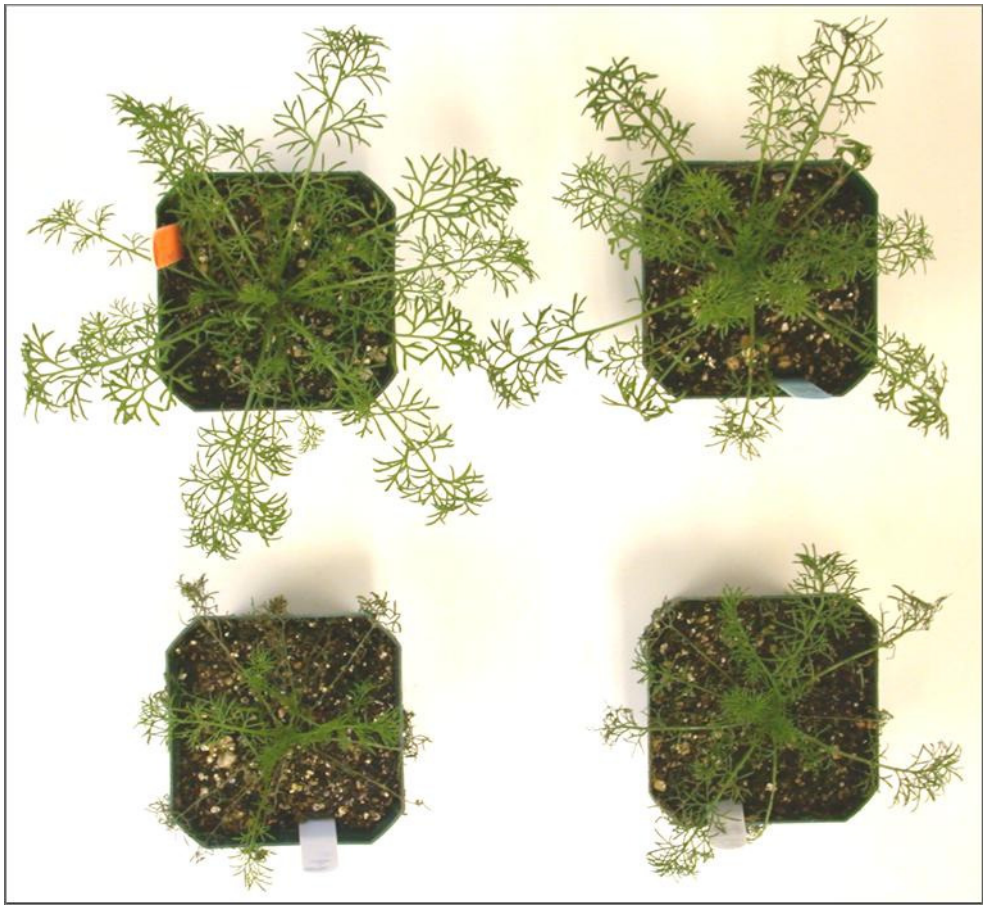

Figure 4.3. Effect of glucose concentration used in the inoculum production medium on efficacy of *C. truncatum* against scentless chamomile. Clockwise from top left: untreated control, 40 g/L, 10 g/L, 5 g/L glucose.

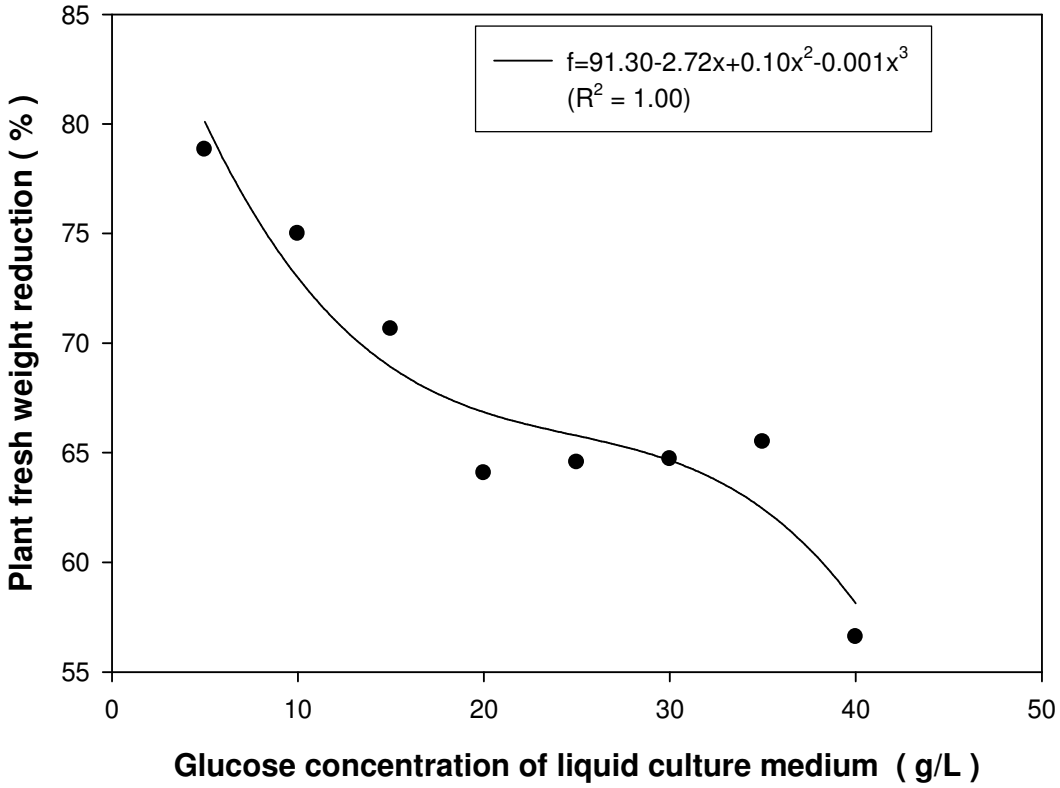

Figure 4.4. Effect of glucose concentration used in the inoculum production medium on efficacy of *C. truncatum* against scentless chamomile. Cubic regression analysis of means of 8 replicates from two combined trials.

4.6. Carbon-to-nitrogen ratio

Data from two trials had non-homogeneous variances, and therefore were analyzed separately. In one trial, when the medium had a C:N ratio of 20:1, significantly more spores were produced compared to other treatments except for the 25:1 ratio (Table 4.8). There were no significant differences in sporulation in the other trial (ANOVA, $P>0.05$).

All bioherbicide treatments caused infection on scentless chamomile plants regardless of the C:N ratio in the medium used to produce the inoculum, with significantly lower fresh weights when compared to the control (Table A.8, Appendix). Spores produced at C:N ratios of 20:1 to 25:1 caused significantly higher fresh weight

reduction of treated plants than spores produced at C:N ratios of 30:1 or 40:1 (Table 4.9).

Table 4.8. Effect of C:N ratio on spore yield

C:N ratio	Spore yield (sp/ml)[a]	
5:1	9.00×10^4	c
7:1	3.72×10^4	c
10:1	3.02×10^4	c
15:1	1.70×10^5	c
20:1	5.04×10^6	a
23:1	1.88×10^6	bc
25:1	3.40×10^6	ab
30:1	1.60×10^5	c
40:1	4.52×10^5	c

[a] Means of 3 replicates from one trial. Means with the same letter(s) are not significantly different (LSD, *P*=0.05).

Table 4.9. Effect of C:N ratio used in the inoculum production medium on efficacy of *C. truncatum* against scentless chamomile

C:N ratio	Fresh weight reduction (%)[a]	
10:1	53.2	ab
15:1	47.2	abc
20:1	55.1	a
23:1	57.5	a
25:1	58.2	a
30:1	40.8	bc
40:1	35.3	c

[a] Means of 8 replicates from 2 trials. Means with the same letter(s) are not significantly different (LSD, *P*=0.05).

4.7. Light/dark conditions

The effect of supplementary lighting on sporulation of *C. truncatum* was not significant (ANOVA, P>0.05). All treatments yielded approximately 1×10^6 sp/ml after 2 wk.

4.8. Fermentation temperature

Comparison of varying combinations of temperatures showed that either an initial or a constant 20°C was more favourable to sporulation than either an initial or constant 12°C or 16°C, while sporulation at 20°C for one week followed by 12°C for one week was similar to 20°C for two weeks, but higher than all other treatments (Figure 4.5).

All bioherbicide treatments caused infection on scentless chamomile plants, with significantly lower fresh weights compared to the untreated control (Table A.9, Appendix). Cooler temperatures (12°C or 16°C), constant or for the 2nd wk only, produced conidia that were significantly more virulent than those produced under the constant 20°C condition, with a greater impact on fresh weight reduction of scentless chamomile (Figure 4.6).

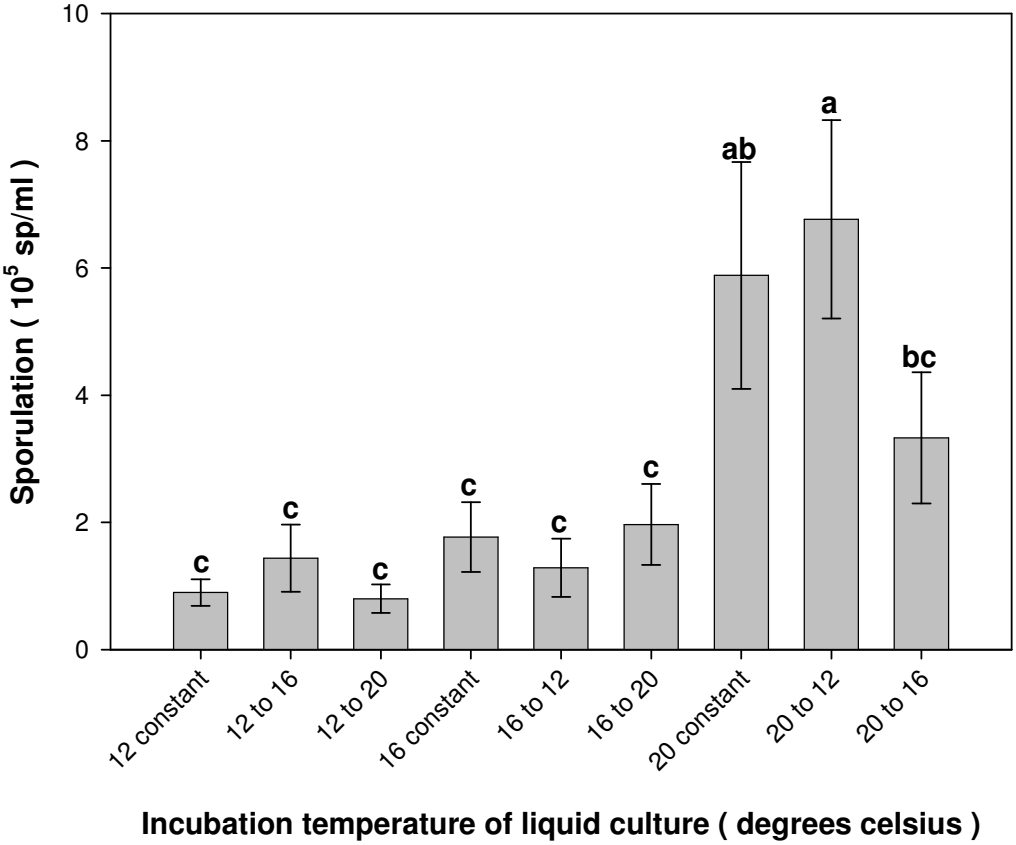

Figure 4.5. Effect of incubation temperature on spore yield. Means of 12 replicates from 3 trials. Bars with the same letter(s) are not significantly different (LSD, P=0.05).

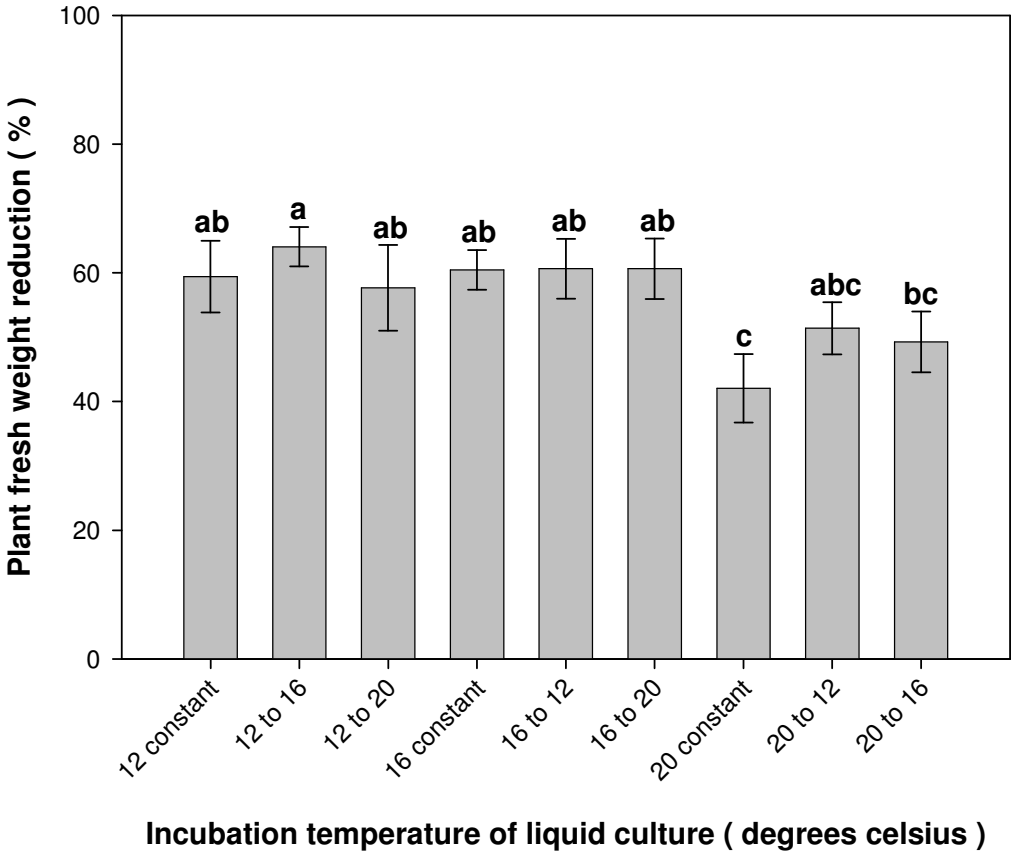

Figure 4.6. Effect of incubation temperature used during inoculum production on efficacy of *C. truncatum* against scentless chamomile. Means of 8 replicates from 2 trials. Bars with the same letter(s) are not significantly different (LSD, P=0.05).

4.9. Medium pH

Regardless of the timing of pH adjustment, a pH of 7.5 was significantly better than 6.5 or 5.5, while a pH of 4.5 was not different from the other treatments (Table 4.10). Medium pH dropped somewhat after autoclaving, but remained fairly constant over the 2-wk duration of experiments (Table A.10, Appendix). All bioherbicide treatments caused infection on scentless chamomile with significantly lower fresh weights compared to the untreated control, however there were no significant differences in weed control efficacy for spores produced in cultures with different pH levels (ANOVA, P>0.05).

Table 4.10. Effect of medium pH on spore yield

	Spore yield (sp/ml)[a]			
Initial pH	Medium pH adjusted before autoclaving		Medium pH adjusted after autoclaving	
4.5	1.52×10^6	ab	5.85×10^5	ab
5.5	6.49×10^5	b	6.00×10^4	b
6.5	8.42×10^5	b	7.86×10^4	b
7.5	2.99×10^6	a	9.30×10^5	a

[a] Means of 8 replicates from 2 trials for each experiment. Means with the same letter(s) within an experiment are not significantly different (LSD, P=0.05).

4.10. Aeration

Sporulation data from two trials showed a similar trend, but had non-homogeneous variances, and therefore were analyzed separately. In the first trial, there was no significant interaction between flask design and shaker speed (ANOVA, P>0.05), whereas the main effect was significant for both factors (ANOVA, P>0.05). In the second trial, there was significant interaction between flask design and shaker speed (ANOVA, P<0.05), and the main effect was significant for shaker speed (ANOVA, P<0.05) but not flask design (ANOVA, P>0.05).

In the first trial, the fungus produced significantly more spores in baffled flasks than regular, and significantly fewer spores at 50 RPM than 150-200 RPM shaker speeds. Overall, sporulation was significantly better in baffled flasks at 200 RPM than other treatments except for 150 RPM (Table A.11, Appendix). In the second trial, the fungus produced significantly more spores at 200 RPM than lower shaker speeds. Overall, sporulation was significantly better in baffled flasks at 200 RPM than other treatments (Table A.12, Appendix).

Biomass data from two trials had homogenous variances, and were combined. There was no significant interaction between flask design and shaker speed (ANOVA, P>0.05), and the main effect was not significant for either factor (ANOVA, P>0.05).

Linear regression analysis suggested a positive effect of shaker speed on sporulation for cultures in both types of flasks. The relationship of shaker speed to biomass was analysed with a quadratic regression (Figure 4.7).

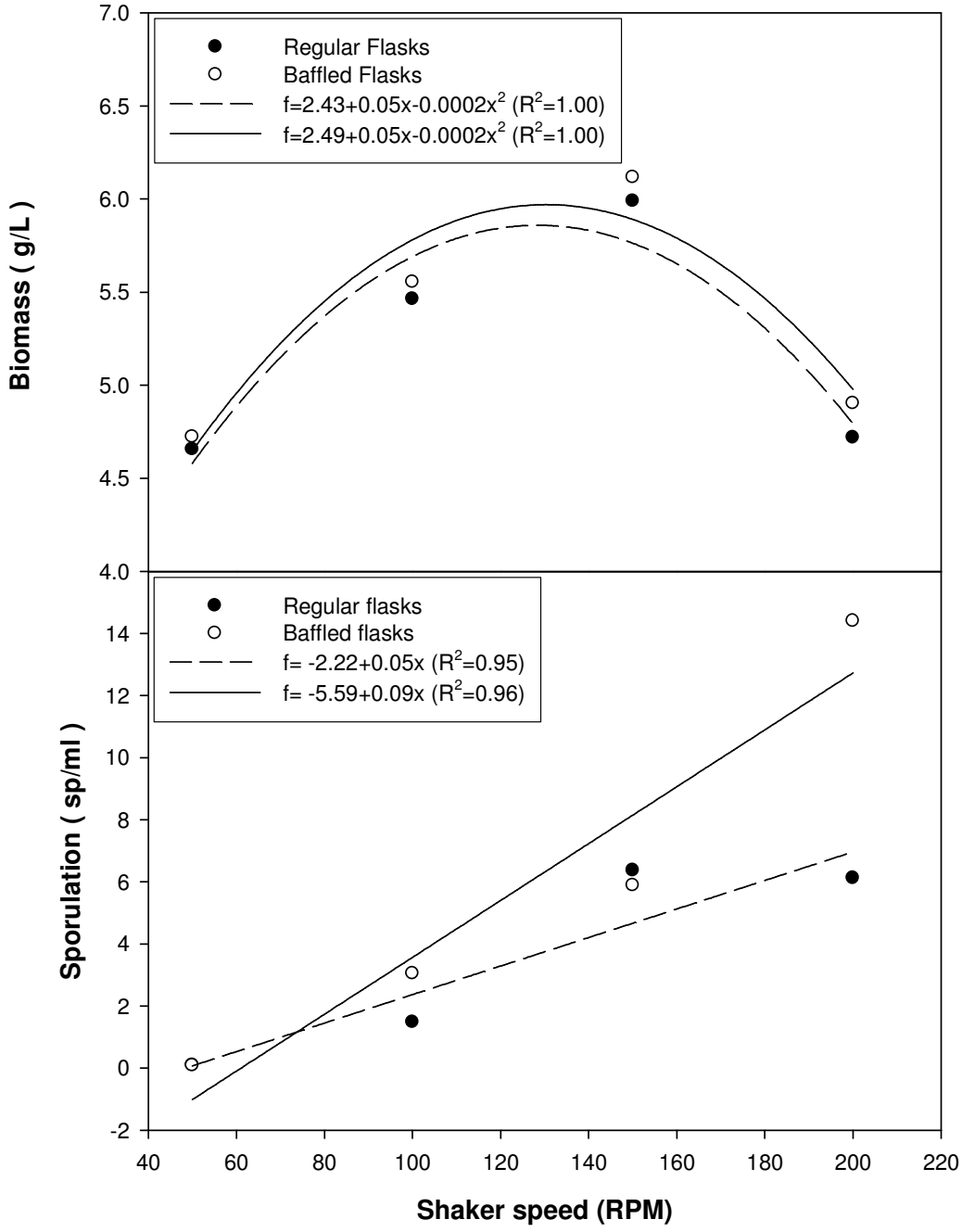

Figure 4.7. Effect of flask type and shaker speed on biomass production and spore yield. Regression analysis of means of 8 replicates from two combined trials.

4.11. Interaction between aeration and glucose concentration

Regression analyses showed that biomass and sporulation data fit linear and quadratic equations, respectively (Figure 4.8). Fungal biomass increased as glucose concentration increased, regardless of aeration conditions. In contrast, the effect of glucose concentration on sporulation depended on the level of aeration used. At low aeration, spore yields changed little with different glucose concentrations in the medium. At high aeration, however, sporulation increased as glucose concentration increased, with the 40 g/L producing the highest number of spores.

Further analysis of sporulation data showed a significant interaction between aeration and initial glucose concentration, and the main effect was also significant for both factors (ANOVA, $P<0.05$). Higher aeration and 40 g/L glucose were more favourable to fungal sporulation than other treatments (Table A.13, Appendix). Similar analysis on biomass data indicated that the interaction between aeration and glucose concentration as well as the main effect of aeration were not significant (ANOVA, $P>0.05$), while the main effect of glucose was significant (ANOVA, $P<0.05$). When the medium contained 40 g/L glucose, a greater amount of biomass was produced than at lower concentrations (Table A.14, Appendix).

Efficacy data indicated that the interaction between aeration and glucose concentration as well as the main effect of aeration were not significant (ANOVA, $P>0.05$), while the effect of glucose concentration was significant (ANOVA, $P<0.05$). All bioherbicide treatments caused infection on the plants, resulting in significantly lower fresh weights when compared to the untreated control (Table A.15, Appendix). The glucose concentration of the medium used to produce the fungal spores had a significant effect on fresh weight of scentless chamomile (ANOVA, $P<0.05$), with inoculum produced at 5 g/L causing plants to have a significantly lower mean fresh weight than inoculum produced at 20-40 g/L. In general, there was a trend for inoculum efficacy to increase as glucose concentration of the production medium decreased, and this was illustrated by fresh weight reduction analysis with a quadratic regression (Figure 4.9).

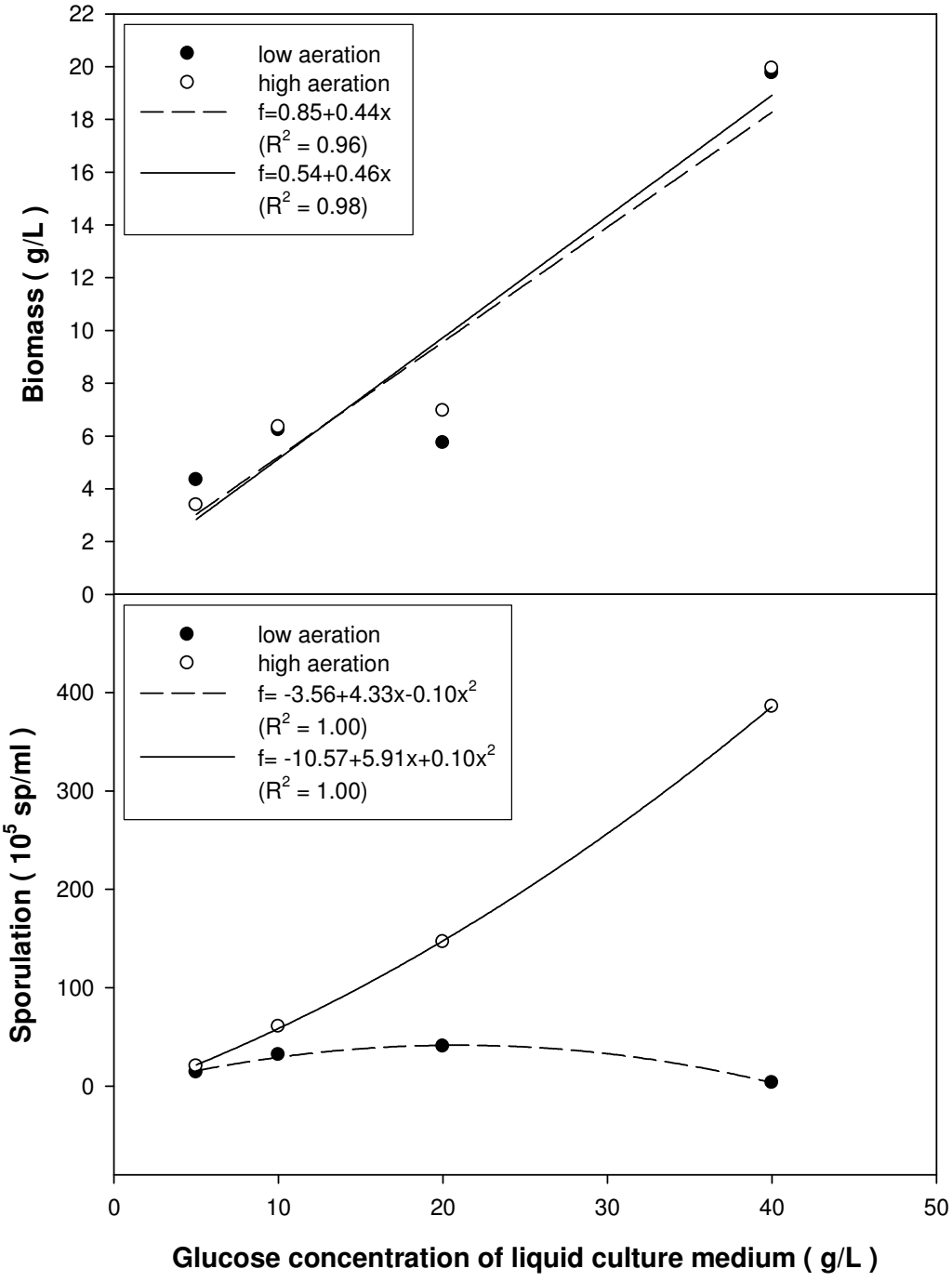

Figure 4.8. Effect of glucose concentration and aeration on biomass production and spore yield. Regression analysis of means of 8 replicates from two combined trials.

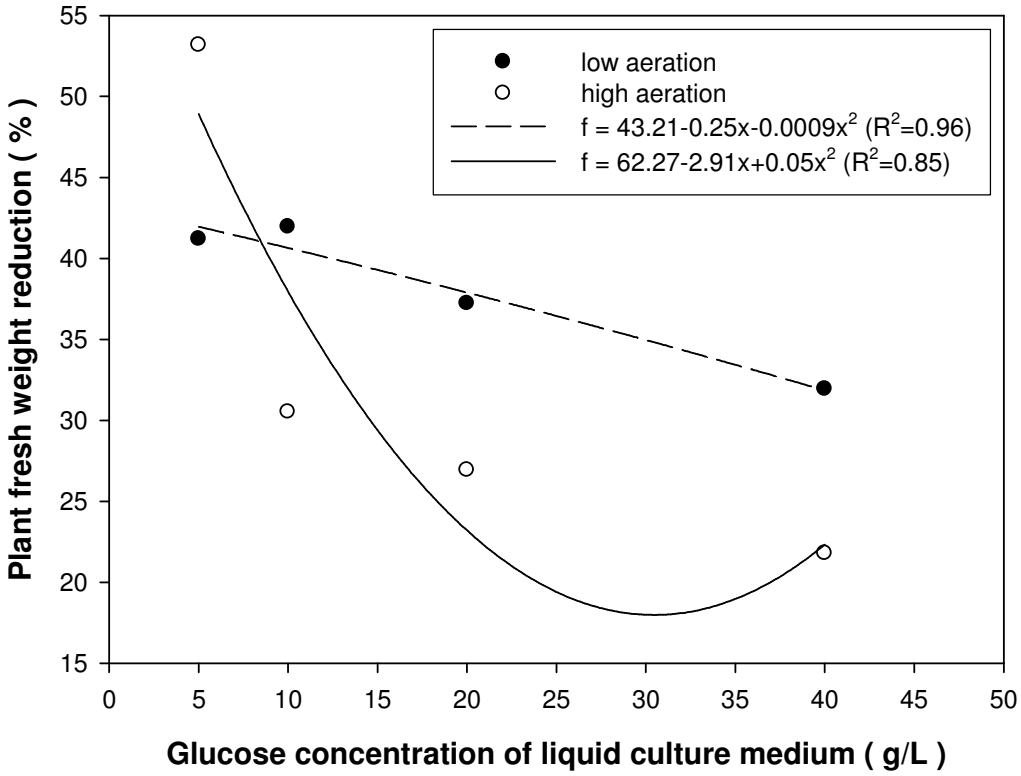

Figure 4.9. Effect of glucose concentration in the DBSM and aeration level used during inoculum production on efficacy of *C. truncatum* against scentless chamomile. Quadratic regression analysis of means of 8 replicates from two combined trials.

Glucose concentration in the liquid medium declined with the progress of fermentation, and often more rapidly after day 8 when fungal sporulation was first observed (Figure 4.10). On day 14 when spores were harvested, the amount of glucose remaining in the medium varied depending on the initial amount added. Little was left in the media that had initially contained 5-10 g/L, regardless of the aeration level. There tended to be more glucose remaining in the media with higher initial glucose concentrations, especially under the lower level of aeration.

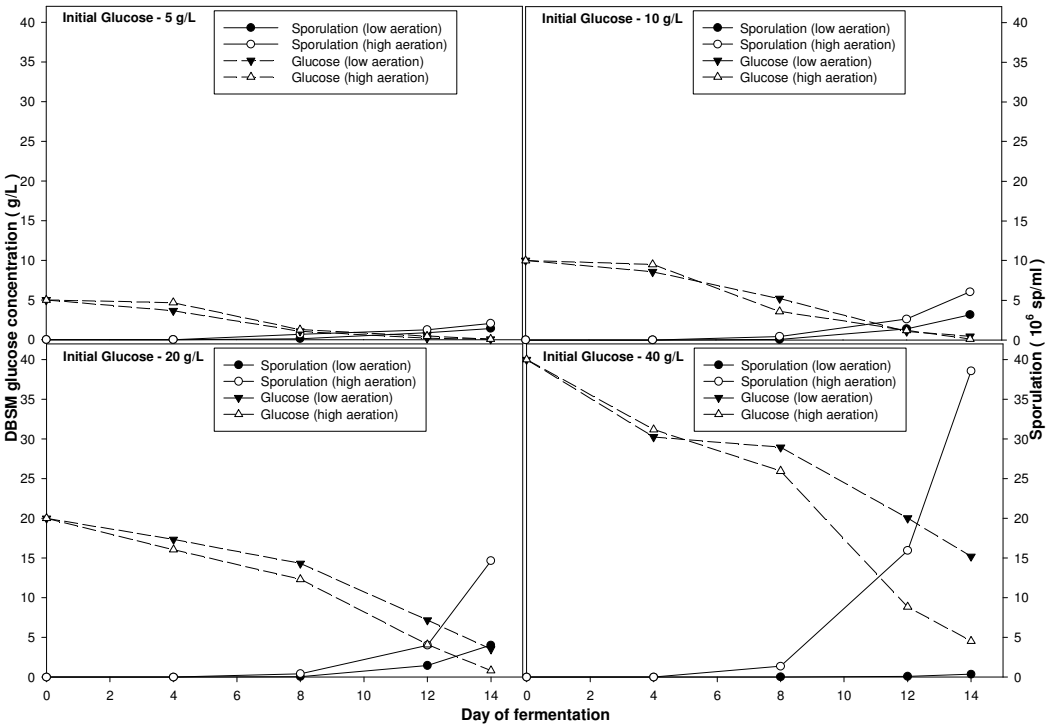

Figure 4.10. Glucose consumption and sporulation of *C. truncatum* during submerged fermentation in media with different glucose concentrations and aeration levels.

The specific spore yields of cultures with 20-40 g/L glucose at high aeration were significantly higher than those of other treatments (ANOVA, $P<0.05$), regardless of whether the specific spore yield was based on initial glucose level or the amount of glucose used during the fermentation (Table A.16, Appendix). The specific biomass yield of cultures with 5 g/L at low aeration was significantly higher than those of other treatments (ANOVA, $P<0.05$), except for 5 g/L at high aeration when the specific spore yield was based on initial glucose level, while specific spore yields based on the actual amount of glucose used during the fermentation were similar for 5-10 g/L and 40 g/L at low aeration and 5-10 g/L at high aeration (Table A.17, Appendix).

There was a significant positive correlation between sporulation and biomass yield as well as biomass and remaining glucose in the media at end of fermentation, while the sporulation and remaining glucose were not correlated (Table 4.11).

Table 4.11. Correlation between sporulation, biomass, and remaining glucose in the DBSM at the end of fermentation

Variables [a]	Pearson correlation coefficient (R^2)	Significant (P=0.05)
Sporulation and biomass	0.43173	Yes
Sporulation and remaining glucose	-0.16005	No
Biomass and remaining glucose	0.55710	Yes

[a] Data from all treatments, including different levels of aeration and initial concentrations of glucose, were used for the analysis.

4.12. Scale-up of production using 20-litre fermentors

The following optimized parameters were determined from previous flask fermentations and selected for the 20-L fermentation experiment. The temperature regime was 20ºC for one week followed by 12ºC for one week. Glucose and casamino acids were used to supply C and N, respectively, with an initial glucose concentration of 5.5 g/L a 23:1 C:N ratio. The pH was adjusted to 7.5 at the start of the run and was monitored but not adjusted after this point.

The dissolved oxygen (dO) reached target levels on day 3 for the 60%-dO fermentor and on day 4 for the 30%- and 10%-dO fermentors (Figure 4.11). The designated dO levels were maintained until day 10, when cultures became progressively more viscous and proper stirring could no longer be achieved at the target dO levels. Stirring was resumed, which resulted in an increase in dO levels in all fermentors. The thickening of cultures was accompanied by polysaccharide production and formation of dark-coloured sclerotia in all fermentors.

Glucose concentration in the media changed little in the first 3 d, but started to decline dramatically after day 4 and dropped nearly to 0 g/L by day 5 for 60% dO, day 6 for 30% dO, and day 7 for 10% dO (Figure 4.12). Biomass increased most rapidly during the first 6 d of fermentation but changed little thereafter except for the culture with 10% dO that reached the maximum level on day 10 (Figure 4.13).

Sporulation was observed on day 4 and reached the maximum between day 8 to 11, depending on the dO level provided (Figure 4.14), with the highest spore counts from the culture under 30% dO. After day 11, spore numbers declined in all cultures, accompanied by a build-up of fungal mycelia on the inner wall of the fermentor. However, spore yields were higher for the last count with each fermentor after cultures were harvested and additional fungal materials were washed from the sides of the wall.

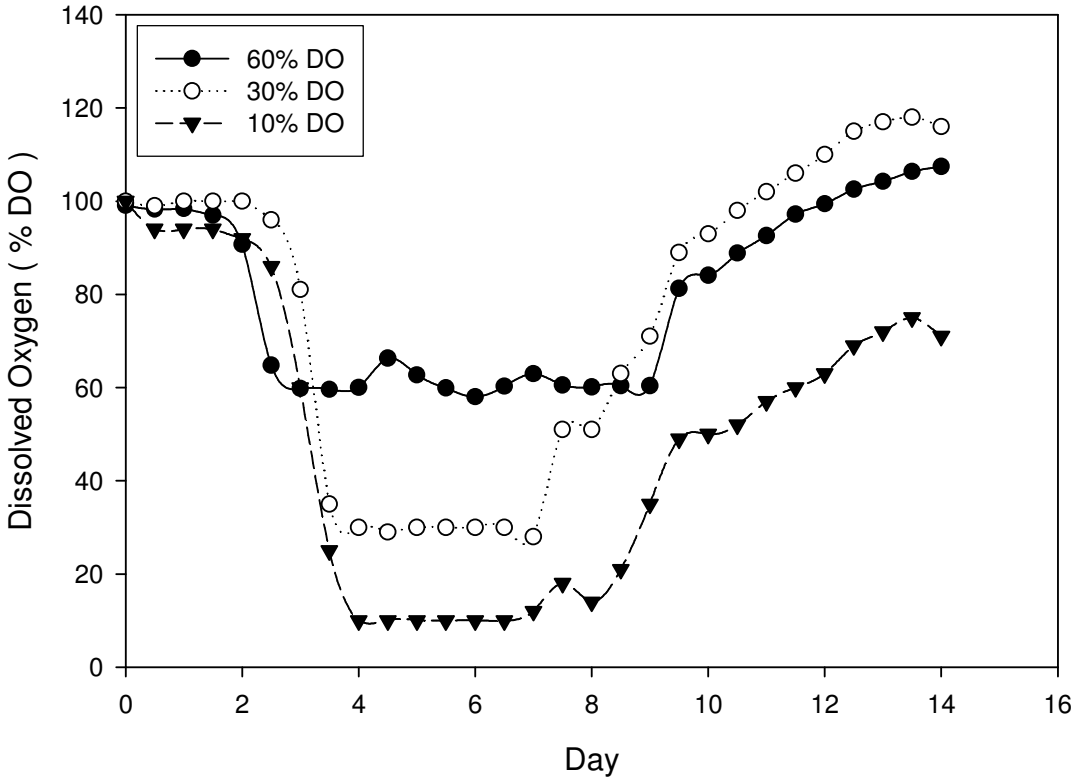

Figure 4.11. Dissolved oxygen levels during fermentation of *C. truncatum*

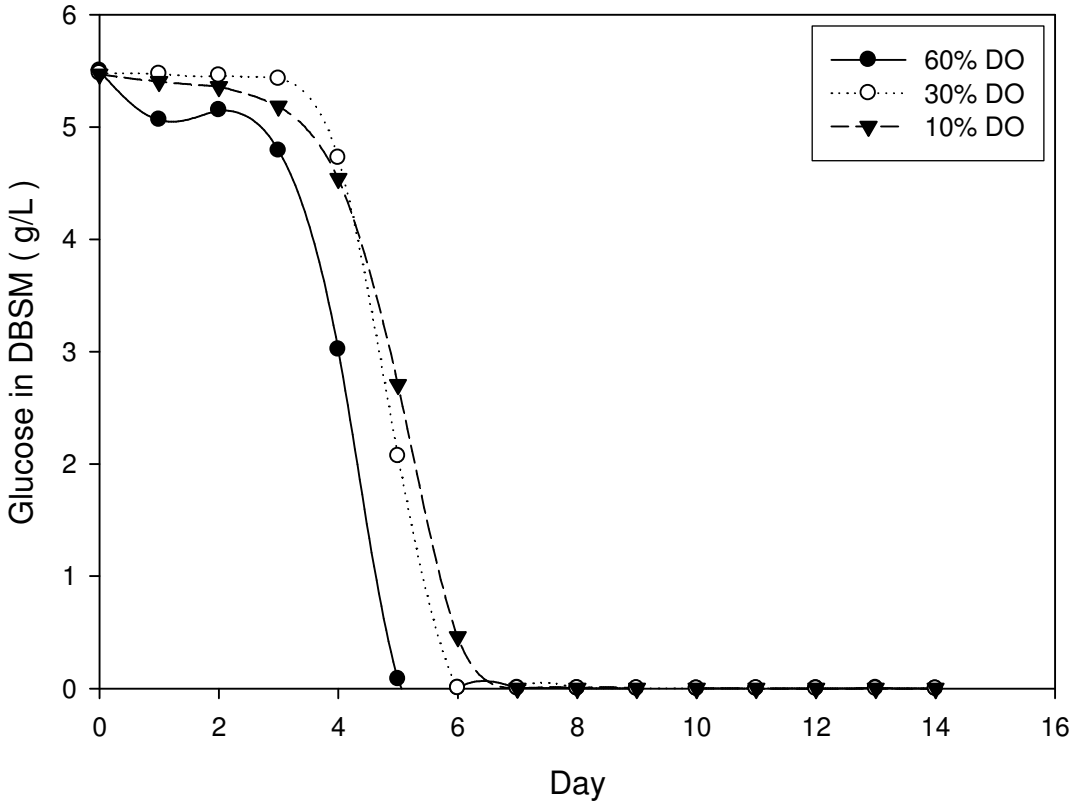

Figure 4.12. Glucose concentration in culture media during fermentation

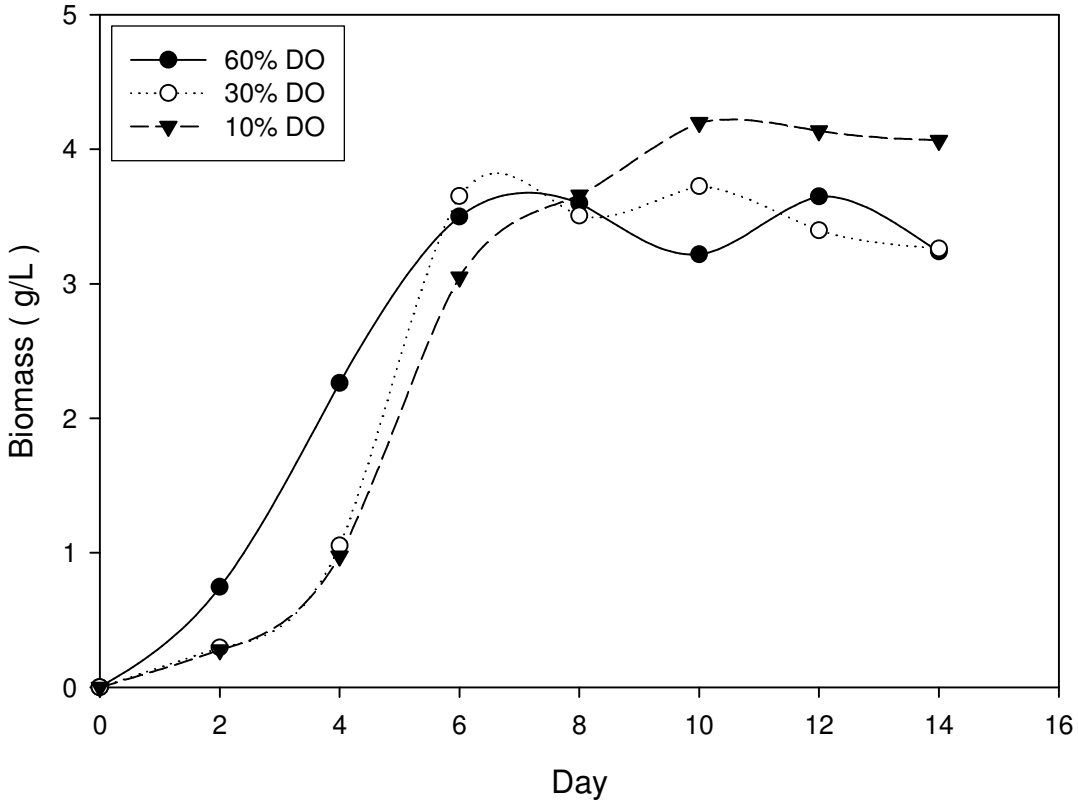

Figure 4.13. Biomass production of *C. truncatum* during fermentation

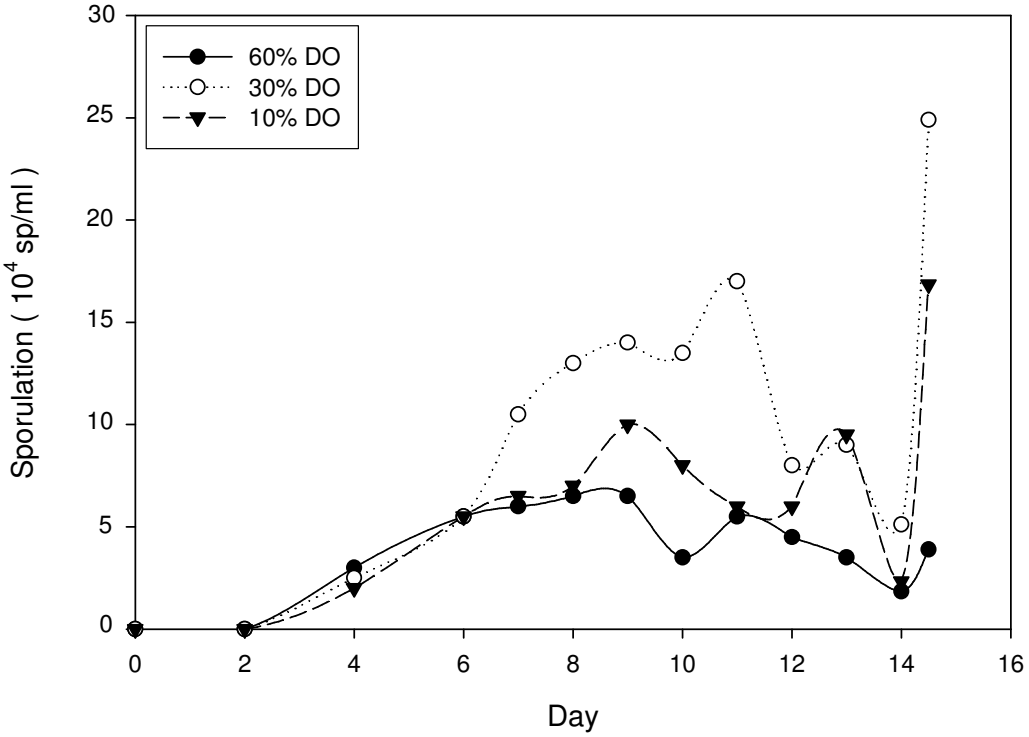

Figure 4.14. Spore production of *C. truncatum* during fermentation

5. DISCUSSION

5.1. Medium type and incubation temperatures

Optimization strategies for bioherbicide production begin with development of a suitable medium for biomass increase and propagule formation (Jackson et al. 1996). Two types of liquid media were selected for this study, based on results of other studies (Graham et al. 2005; Jackson and Bothast 1990; Jackson et al. 1996; Morin et al. 1990; Zhang et al. 2001), to determine sporulation potential for this *C. truncatum* strain at different incubation temperatures.

Spores of *C. truncatum* could be produced on a solid V8 medium (Graham et al. 2005) and V8 media have been used to produce conidia of other fungal species in liquid cultures (Morin et al. 1990). However, V8 juice was inferior to other ingredients for sporulation of other *C. truncatum* isolates (Jackson and Bothast 1990; Jackson et al. 1996), and is generally not considered practical for mass production of fungal inoculum because it is not available as a bulk commodity and is too expensive for large-scale commercial fermentation (Yu et al. 1997). Unconsumed materials may also be problematic for product recovery and effluent treatment (Stanbury et al. 1995a). Nevertheless, the liquid V8 medium supported fungal growth and sporulation in this study.

The defined basal salt medium (DBSM) was developed for producing inoculum of another *C. truncatum* strain (Jackson and Bothast 1990; Jackson et al. 1996). With addition of C and N sources, DBSM provided nutritional components that supported growth and sporulation of *C. truncatum* in the current study, and yielded more spores than did V8 under most conditions, suggesting superiority for investigating fungal sporulation in liquid cultures (Table 4.1). Because of its defined elements, this medium provided a useful platform for studying the liquid fermentation process and was easily manipulated to further define the requirements for sporulation.

Defined media usually give more reproducible results than complex media, and therefore spores, biomass, and/or product yields may be more predictable when

individual parameters are being investigated (Davis and Blevins 1979). In industry, however, defined media are rare in large-scale fermentations due to cost considerations for multiple ingredients (Dahod 1999; Davis and Blevins 1979). Frequently, once optimal nutritional elements are determined, complex substrates containing similar critical elements from less expensive sources are sought as substitutes.

Media may also have an effect on fungal growth patterns in submerged cultures. Rich, complex media usually favour a dispersed form of growth, while pellet formations tend to occur in chemically defined media (Stanbury et al. 1995b). This corresponds to the differences in mycelial growth observed with the fungus in this study. It produced thicker masses of mycelium in the V8 but more distinctive mycelial balls in the DBSM. The latter structure may have an increased surface area for fungal sporulation. Morphological differences aside, biomass production was significantly higher at 16-22ºC in DBSM and significantly lower at 28ºC in V8 compared to other treatments (Table 4.2).

This *C. truncatum* strain produced fewer spores and less biomass at 28ºC than at 16-22ºC. Sporulation of C. *truncatum* can be affected by incubation temperature (Chongo and Bernier 2001) but the preference for cooler temperatures by this strain seems rather unique and has not been seen for other *C. truncatum* strains and/or other *Colletotrichum* species (Jackson and Bothast 1990; Olufolaji 1994).

Because there was no significant effect on sporulation after 7 d, and sporulation continued to increase after 10 d, it was decided that spore yields would be measured after 14 d for the subsequent experiments. DBSM was selected as the liquid medium, for following experiments, and a temperature of 16ºC was used.

5.2. Initial inoculum concentration

Initial inoculum concentrations can affect fungal sporulation in liquid cultures (Monaghan et al. 1999), and a range from $1x10^4$ sp/ml to $1x10^7$ sp/ml has been reported with different studies (de la Torre and Cardenas Cota 1996; Jackson et al. 2004; Li and Holdom 1995; Morin et al. 1990; Thomas et al. 1987; Urquhart et al. 1994; Vega et al. 2003; Vidal et al. 1998; Zhang et al. 2001). For example, Slininger *et al.* (1993) used an initial concentration of $1x10^4$ sp/ml for fermentation of *C. truncatum*, whereas initial

concentrations five times greater than this were used to produce conidia of similar fungal organisms in several separate studies (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002; Montazeri et al. 2003). For the fungus *Penicillium oxalicum* Currie & Thom., an initial concentration of $1x10^7$ sp/ml was used (Pascual et al. 1997), which was the highest level reported for similar studies in the literature. When the initial inoculum concentration is too high, an auto inhibition or crowding effect may occur, preventing spore germination and, consequently, biomass increase in the liquid culture. This effect has been demonstrated for fermentation of *Penicillium bilaji* Chalab. and *C. gloeosporiodes* under initial inoculum concentrations above $1x10^6$ sp/ml (Cunningham et al. 1990). These results may explain why higher initial inoculum concentrations in this experiment gave rise to fewer *C. truncatum* spores and less biomass (Figure 4.1, Table 4.3). Based on the present study, the optimal level of *C. truncatum* inoculum at the beginning of fermentation should be between $1x10^3$ and $1x10^4$ sp/ml, which is lower than levels reported in most of the previous studies in which *C. truncatum* conidia were produced.

In this study, the volume of seed inoculum added to the fermentation medium ranged from 0.01% to 10% (v/v). In comparison, industry usually uses a seed volume of 1-15% for liquid fermentation. Silman and Nelsen (1993) added seed inoculum at about 2.5% (v/v) for fermentation of *C. truncatum*, whereas Zhang et al. (2001) used 0.5% seed-inoculum volumes for production of *P. tabacinum* in liquid cultures. A concern with high seed inoculum volume is substantial dilution of the medium, which potentially affects spore yield and potency. In the current study, this factor was avoided by preparing highly concentrated seed inocula (1×10^7 sp/ml) in the same liquid medium used for fermentation. As a result, an aliquot of 100-µl of seed suspension was sufficient for initiating shake-flask cultures with an optimal initial inoculum concentration of 1×10^4 sp/ml.

5.3. Carbon sources

In this study, DBSM supplemented with sucrose or glucose was superior to lactose, cellulose, fructose, and molasses in terms of fungal sporulation. Inoculum produced in glucose or fructose was superior to maltose, while similar to sucrose and

trehalose in terms of efficacy against scentless chamomile (Table 4.4, Table 4.5). Glucose was chosen for use in the medium for future experiments for the additional reason that it was compatible with the method used to monitor C exhaustion.

Jackson and Bothast (1990) found that sporulation in cultures supplemented with acetate, galactose, citrate, and cellulose was generally lower than with glucose, sucrose, fructose, glycerol, and corn starch, with acetate being significantly poorer than all other sources tested. Glucose was chosen as the C source in their defined medium also because it was easy to measure and produced high conidial yields in liquid cultures (Jackson and Bothast 1990). Other authors also used glucose for production of *C. truncatum* (Montazeri and Greaves 2002). In addition, glucose has been used as a C source in media for sporulation of several fungal biopesticide agents, including *B. bassiana, M. anisopliae, P. fumosoroseus*, *P. tabacinum,* and *Tilletiopsis* Derx spp., under submerged fermentation conditions (Cliquet and Jackson 2005; Jackson et al. 2004; Thomas et al. 1987; Urquhart et al. 1994; Vega et al. 2003; Zhang et al. 2001). Glucose and fructose are monosaccharides; trehalose, sucrose, lactose, and maltose are disaccharides; starches and cellulose are polysaccharides. Because glucose is available to the fungus in monomeric form, it can be used immediately, while polymers must be hydrolysed by the fungus before they can be used (Ooijkaas et al. 1998).

Sucrose was used as the C source in a modified Richard's medium for liquid production of *C. coccodes* spores (Yu et al. 1997). In a separate study, although media containing sucrose supported only moderate sporulation of *P. tabacinum*, these spores were more efficacious against false cleavers, reducing plant dry-weight by more than 99% compared to untreated controls. When galactose was used as the C source, however, spore yields were higher but the inoculum was less virulent, reducing the dry-weight by only 62% (Zhang et al. 2001).

Varying effects of other C sources on fungal sporulation have been reported with other biopesticides for both solid and liquid medium production. In a study of eight different C sources in solid media for production of *F. moniliforme* var. *subglutinans,* Bolkan (1982) found that the fungus produced the most dense mycelium in media containing maltose, lactose, or glucose, while it yielded highest numbers of spores in media using sucrose, fructose, or soluble starch as a C source. Another species of

Fusarium required 2 % cellulose as the sole C source in the presence of 0.5-0.75% yeast extract for sporulation (Ogel et al. 1994). Starch was preferred over glucose for spore production of *C. minitans,* a biocontrol agent of *Sclerotinia sclerotiorum* (Lib.) de Bary*,* because substrate inhibition of sporulation occurred when glucose was used (Ooijkaas et al. 1998).

5.4. Nitrogen sources

In this study, the complex N sources casamino acids, casein enzymatic hydrolysate, and cottonseed hydrolysate resulted in the highest sporulation in liquid media (Table 4.6). Cottonseed hydrolysate was deemed unsuitable however, because cultures were viscous and spores could not be harvested adequately. In another study, complex N sources were superior for sporulation of a different strain of *C. truncatum* and cultures amended with casamino acids resulted in the highest conidial yields among 18 N sources tested (Jackson and Bothast 1990). Consequently, casamino acids were selected by these researchers as the N source in the DBSM because they were vitamin-free, relatively consistent in composition compared to many other proteinacious sources, and high yielding of conidia in liquid cultures (Jackson and Bothast 1990). For similar reasons, casamino acids were also selected as the N source for further experiments in the present study. Yu et al. (1997) reported that either casamino acids or soy proteins could be used to replace the more expensive V-8 juice in modified Richard's medium for production of *C. coccodes* without sacrificing conidial yields. In contrast, inorganic N sources generally resulted in poor sporulation of *C. coccodes* (Yu et al. 1997). Casamino acids have also been used as an N source for sporulation of other fungal biopesticide species in submerged cultures, such as *P. fumosoroseus*, *B. bassiana,* and *M. anisopliae* (Cliquet and Jackson 2005; Jackson et al. 2004; Vega et al. 2003).

In addition to affecting the yield, N sources may have an impact on other attributes of fungal sporulation. Further studies were conducted by Jackson *et al*. (1993) to compare N sources in DBSM using 8g/L of glucose at a constant C:N ratio of 15:1 for sporulation of *C. truncatum*. Urea and individual amino acids delayed sporulation while casamino acids with a defined set of amino acids caused rapid and optimal sporulation.

While both organic and inorganic N sources supported sporulation of *C. truncatum,* yields were higher with the complex organic casein enzymatic hydrolysate than the inorganic sources ammonium nitrate and potassium nitrate. Sporulation with casein enzymatic hydrolysate was similar to other complex sources cottonseed hydrolysate and casamino acids, but spore yields with casein enzymatic hydrolysate were higher than other organic sources tested, including tryptone and 2 amino acids L-leucine and L-glutamic acid. Potassium nitrate was used for production of *B. bassiana* spores (Thomas et al. 1987). A moderate number of *P. tabacinum* spores were produced in a medium containing potassium nitrate, but these spores incited 100% dry-weight reduction of false cleaver seedlings, whereas a medium with corn gluten meal as the N source produced higher spore numbers but only 80% dry-weight reduction (Zhang et al. 2001). This indicates that the N source can also affect the efficacy of fungal inoculum produced. This effect was not identified in the current study, possibly because only two treatments were available (casamino acids and casein enzymatic hydrolysate) for plant inoculation, and overall efficacy was low compared to other trials (Table 4.7).

5.5. Glucose concentration

With increasing concentration of glucose in the medium, biomass and spore yield of *C. truncatum* also increased (Figure 4.2). Within the range of glucose concentrations tested, maximum sporulation was achieved at 35-40 g/L of glucose. Jackson *et al.* (1990; 1996) observed maximum sporulation of a different strain of *C. truncatum* at slightly lower glucose concentrations (10-30 g/L) and their strain did not sporulate at concentrations higher than 50 g/L glucose, but rather promoted production of highly melanized hyphal aggregates identified as microsclerotia (Jackson and Bothast 1990; Jackson et al. 1996). This type of structure was of little interest for biocontrol of scentless chamomile due to poor infection efficiency on the foliage (Peng (personal communication, 2003)), although it was observed along with fungal mycelium and conidia during submerged fermentation throughout this study.

Fungal sporulation in cultures of *C. coccodes* increased as sucrose concentration increased, however the specific spore yield, based on the conversion of medium raw materials to spores, was constant (Yu et al. 1998). Specific spore and biomass yields

calculated for *C. truncatum* in this study also showed no significant differences for most treatments within the glucose concentration range tested, suggesting additive effects of C concentration. In the case of *C. coccodes*, the C:N ratio interacted with the effect of C concentration on mycelial biomass; at low C:N ratios, increased C concentration caused specific mycelial biomass to increase significantly, whereas at high ratios C concentration had no effect or even a negative effect (Yu et al. 1998). This type of interaction was not investigated in this study, but Yu's results indicate that the effect of C concentration can be complex and may be influenced by the C:N ratio of the medium.

In the current study, *C. truncatum* spores produced under high glucose concentrations tended to be less efficacious against scentless chamomile than those produced under low glucose conditions for control of scentless chamomile, and the difference was significant between the lowest (5 g/L) and highest (40 g/L) concentration treatments tested (Figure 4.3, Figure 4.4). Excess C may be converted to lipid by the fungus during fermentation, decreasing protein content in fungal spores, which may lead to lower conidial germination and bioherbicidal efficacy of the inoculum (Jackson et al. 1996). Increased protein content was associated with increased germination rates and appressorial formation of a *C. truncatum* strain (Jackson and Schisler 1992). The importance of C concentration to biocontrol efficacy was also observed with *P. tabacinum* on false cleavers, in which media with 12.6 g/L C produced the highest number of spores as well as demonstrating the greatest level of weed control efficacy when compared to media with lower or higher initial C concentrations (Zhang et al. 2001).

Although higher glucose concentrations in this study generally increased the yield of fungal spores, the negligible specific spore yield and potential negative impact on bioherbicidal efficacy should not be overlooked. Results of the current study indicate that a glucose concentration that achieves a high yield of *C. truncatum* spores with minimal sacrifice of biocontrol efficacy on scentless chamomile must be selected. A glucose concentration of 5-10 g/L would be optimal for the medium composition under the conditions used in this experiment.

5.6. Carbon-to-nitrogen ratio

A C:N ratio between 20:1 and 25:1 in the medium showed significantly higher sporulation than other C:N ratios in one of the trials (Table 4.8), which is different from other studies on the production of *C. truncatum* conidia. For example, Jackson and Bothast (1990) found that media with the C:N ratio of 15:1 were consistently superior in supporting sporulation of *C. truncatum* compared to media with a ratio of 40:1 or 5:1. Montazeri and Greaves (2002) reported similar results with a *C. truncatum* strain that produced significantly fewer spores in a medium with a 40:1 C:N ratio than in media with lower C:N ratios.

The efficacy of spores produced in media with C:N ratios between 20:1 and 25:1 was significantly higher than that at higher ratios (Table 4.9). Several authors have suggested that *C. truncatum* conidia produced in a medium with a C:N ratio around 10:1 tend to incite more severe disease than conidia produced at 30:1, which was the C:N ratio that generally produced the highest spore yield in submerged fermentation (Bothast et al. 1993; Jackson and Bothast 1990; Jackson et al. 1996; Schisler et al. 1995). However, the lower efficacy of fungal inoculum produced under higher C:N ratios was also shown to be potentially reversible. For example, Bothast *et al.,* (1993) and Schisler *et al.,* (1995) showed that efficacy of *C. truncatum* conidia produced in a medium with a 30:1 ratio could be enhanced to levels comparable to those produced at 10:1 by adding pregelatinized starch after fermentation to conidial formulations prior to treatment.

C:N ratios of 10:1, 30:1, and 80:1 were evaluated for liquid *C. truncatum* cultures and conidia from the 10:1 ratio medium had significantly higher protein but lower lipid content when compared to those produced at 30:1 or 80:1 (Jackson and Schisler 1992). The higher protein content in the fungal inoculum was associated with increased spore germination and appressorial formation on hemp sesbania. Further experiments with the same organism revealed that C:N ratios between 20:1 and 15:1 were optimal for sporulation (Jackson and Schisler 1992), but spores produced at 5:1 showed higher germination and caused more lesions on hemp sesbania than those produced under higher C:N ratios (Montazeri and Greaves 2002). In their case, as C:N ratio increased, relative glucose content in the conidia increased, but the portion of trehalose and total polyols (including glycerol and mannitol) decreased. It is not clear how these changes

affect the function of fungal inoculum since the composition of extracellular carbohydrates and proteins of conidia was not substantially affected (Montazeri et al. 2003). A similar effect of the C:N-ratio was also observed with production of *C. coccodes* conidia, in which the highest spore yield and efficacy against velvetleaf were achieved with a medium C:N ratio of 10:1 while media with 5:1 or 20:1 ratios were substantially less favourable (Yu et al. 1998).

Results from various studies indicate that developing media solely to maximize yields of fungal biomass or spores is not justified for production of bioherbicide agents because of the potential negative impact of C concentration and C:N ratio on inoculum efficacy. The balance of these nutritional elements in a liquid medium may also affect shelf-life of an inoculum (Schisler et al. 1995). A C:N ratio of 20:1-25:1 would be optimal for the medium composition under the conditions used in this experiment.

5.7. Light/dark conditions

Some species of fungi do not require light for growth or sporulation (Bolkan et al. 1982; de la Torre and Cardenas Cota 1996; Pascual et al. 1997; Winder and Van Dyke 1990), while others sporulate under certain light regimes or require specific light/dark cycles to sporulate (de la Torre and Cardenas Cota 1996; Figliola et al. 1988; Jamil and Nicholson 1989; Urquhart et al. 1994; Walker and Riley 1982; Zhang et al. 2001). Because no significant differences in sporulation were observed between different light/dark conditions in this study, it appears that this strain of *C. truncatum* does not require light for growth or sporulation, nor is it inhibited by light exposure. These results correspond with other studies which have shown no significant differences in fungal growth and sporulation when different regimes of fluorescent light as well as total darkness were used (Bolkan et al. 1982; Zhang et al. 2001). Other *Colletotrichum* species have also been grown in liquid cultures in the dark for sporulation (Montazeri and Greaves 2002; Montazeri et al. 2003). It is more advantageous to select an organism that does not require stringent light regimes for sporulation in submerged cultures, as meeting those requirements can be a challenge for large-scale production facility. Subsequent experiments in this study were incubated in the dark.

5.8. Fermentation temperature

There was an indication based on earlier trials that this strain of *C. truncatum* preferred a cooler temperature for sporulation (Table 4.1), which may differ from other *Colletotrichum* species/strains studied. For example, spores of *C. coccodes*, the bioherbicide agent for velvetleaf, were produced in liquid cultures at 23 or 24ºC (Yu et al. 1997; Yu et al. 1998). Spores of a different strain of *C. truncatum* were produced in submerged fermentation at 28°C (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002; Montazeri et al. 2003; Slininger et al. 1993). In contrast, the *C. truncatum* strain used in this current study produced significantly lower biomass and was almost non-sporulating at 28°C (Table 4.1, Table 4.2). Microorganisms isolated from cold regions may have lower optimal ranges of temperature than those from warmer areas (Dhingra and Sinclair 1995). The preference for a higher fermentation temperature by the *C. truncatum* strain used to control hemp sesbania is likely related to the fact that this organism was isolated from the southern US (Boyette et al. 1991), a much warmer region than the Canadian prairies (Environment Canada 1993; Fung 1999) where the current biocontrol agent was discovered. An incubation temperature of 20°C has been used for spore production of several other fungal biocontrol agents, including *Tilletiopsis* spp., *C. minitant*, *P. tabacinum*, and *C. truncatum* (Montazeri and Greaves 2002; Ooijkaas et al. 1998; Urquhart et al. 1994; Zhang et al. 2001).

In this study, cultures that were incubated at 20°C constantly or initially (20°C 1st wk, 12°C for the 2nd wk) produced a greater number of spores than those at 12° or 16°C for 2 wk (Figure 4.5). Lowering the temperature from 20°C to 12°C after 1 wk may have applied a shock to the culture, which was also used to trigger sporulation of *P. fumosoroseus* (de la Torre and Cardenas Cota 1996). The benefit appeared insignificant in this study because the treatment did not increase the sporulation when compared to the yield under constant 20°C. However, there may be a practical advantage to using a variable regime with a higher initial temperature that supports rapid mycelial growth followed by a lower temperature that stimulates fungal sporulation, and another factor to consider is the efficacy of inoculum produced under different temperatures. Cultures incubated at a constant 12°C or 16°C showed a higher level of efficacy against scentless

chamomile than those incubated at a constant 20°C (Figure 4.6). Based on these observations, a regime with 20°C for the 1st wk followed by 12°C for the 2nd wk, may offer a compromise between optimal sporulation and inoculum efficacy of this fungus in submerged cultures, and as such was selected for subsequent experiments in this study.

5.9. Medium pH

Medium pH may be adjusted before or after autoclaving (Dhingra and Sinclair 1995), and both of these timings of adjustment were tested to determine the effect of initial medium pH. When the pH was adjusted before autoclaving, the DBSM turned brown during the autoclaving process and this was likely due to a reaction between sugars and ammonium ions or amino acids that formed dark nitrogen-based compounds (Stanbury et al. 1995a). This color change, however, did not occur in media with a pH lower than 5.5, because the chemical reaction rate and equilibrium are pH dependent (Dahod 1999). This reaction may inhibit growth of some microorganisms (Stanbury et al. 1995a) but appeared to have no effect on mycelial growth or fungal sporulation in this study. Fungal sporulation in this study was greater at pH 7.5 than at pH 5.5 or 6.5, regardless of the timing of pH adjustment (Table 4.10). The pH in all other experiments was adjusted prior to autoclaving, and a pH of 7.5 was used.

The optimal pH found within the range tested for this strain of *C. truncatum* was similar to the pH required for conidiation of *C. orbiculare* that causes burr anthracnose (Templeton 1992) but higher than that required for conidial production in other colletotrichum bioherbicide agents. For example, the medium used to produce *C. gloeosporioides* f. sp. *aeschynomene* was adjusted to pH 6 (Templeton 1992), whereas the medium for *C. coccodes* sporulation was adjusted to an initial pH of 5.0 or 5.5 (Yu et al. 1997; Yu et al. 1998). Furthermore, in production of a different isolate of *C. truncatum*, liquid media were adjusted to pH 5.0 to 5.5 (Bothast et al. 1993; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002; Montazeri et al. 2003; Schisler et al. 1995). These levels were monitored and maintained throughout the fermentation process. In contrast, initial adjustment of medium pH used in the current study appears sufficient because pH changed very little during the two weeks of fermentation (Table A.10, Appendix).

Although higher pH was more favourable for sporulation, there may be practical reasons for using lower initial pH to reduce the probability of bacterial contamination, which proved to be an issue throughout this study. *Chondrostereum purpureum* (Pers.) Pouzar mycelia were produced in liquid cultures at an optimal pH range of 5-7; the lower end of the range was frequently used to prevent bacterial contaminants from causing problems during fermentation (M.D. de Jong, C.J.E.A. Bulder, C.A.G.M. Weijers, P.C. Scheepens (unpublished work, 1982)). In a different study, there were no significant differences in blastospore production of *P. fumosoroseus* when fermentation was conducted within a range of pH between 3.5-6.5, but pH 4.0 was selected for production in non-sterile portable fermentation equipment, because low pH reduces the competition from bacterial contaminants (Jackson et al. 2004). If this type of production was desired, a pH of 4.5 may be suitable, as sporulation in the current study was not significantly different than that in the pH 7.5 medium. A higher range of pH could also be tested in future studies to confirm if pH 7.5 is optimal.

5.10. Aeration

Oxygen supply is essential for germination of *C. truncatum* conidia (Slininger et al. 1993), and when deficient, a microorganism may yield less biomass and spores during fermentation or may die (Hosobuchi and Yoshikawa 1999). Aeration was expected to be a critical factor in the present study, but it was difficult to control precisely in flask fermentation. For shake cultures, baffled flasks tend to encourage greater turbulence, consequently increasing the surface area for air transfer and oxygen diffusion into liquid media. A higher concentration of dissolved oxygen in the medium can have significant effects on fungal biomass and sporulation. In a preliminary experiment, sporulation of this *C. truncatum* strain was significantly higher in baffled flasks than in regular Erlenmeyer flasks when the fungal cultures were incubated on the same shaker at 150 RPM (results not shown). Oxygen diffusion into liquid media can also be increased by higher shaker speeds which, when coupled with baffled flasks, creates a broader range of oxygen concentrations for studies using shake cultures. Based on this rationale, baffled flasks and increasing shaker speeds were used in this study as one of the means to enhance aeration.

This study showed a positive effect of baffled flasks and higher shaker speeds on fungal sporulation (Figure 4.7). Although the regression model suggested that the conidial yield would continue to increase with increased aeration, there is likely a technical limitation with the equipment, as a shaker speed of 300 RPM was problematic and caused mycelium to grow more on the sides of flasks than in the medium. As a result, there was little biomass or sporulation in the medium and the treatment was abandoned (results were not included). Conditions favouring fungal growth on flask walls result in physiologically different fermentations simultaneously in the same vessel and are inherently difficult to repeat or scale-up (Hilton 1999). In other studies, shaker speeds ranging from 50 to 300 RPM have been used for sporulation of another *C. truncatum* strain (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002), *C. coccodes* (Yu et al. 1997) and many other fungal species under submerged conditions. For greater aeration, forced air plus agitation may be needed to optimize oxygen supply in liquid fermentation where the oxygen transfer can be regulated by measuring dissolved oxygen (dO) concentration and adjusting agitation speed and/or forced-air pressure (Hosobuchi 1999). However, there may also be an upper limit for oxygen supply, above which fungal sporulation may be decreased due to oxygen toxicity. This effect was observed with a strain of *C. truncatum* when a dO of 75% of maximum inhibited fungal sporulation (Slininger et al. 1993).

For shake-flask experiments, it would be best to use baffled flasks at 200 RPM shaker speed to achieve high sporulation, and it is concluded that aeration is an important factor for sporulation of this strain of *C. truncatum*.

5.11. Interaction between aeration and glucose concentration

Glucose concentration had a more pronounced effect on sporulation at higher aeration levels (Figure 4.8). Although the fungal biomass also increased with higher initial glucose levels, the trend was independent of the aeration level and there was no significant interaction between the two factors. These results indicated that yields of fungal biomass and sporulation were increased at a glucose concentration of 40 g/L, but high aeration was the key for increased spore production.

Similar to the previous glucose concentration experiment in this study, there was a trend for inoculum efficacy to increase as glucose concentration of the production medium decreased (Figure 4.9), indicating that inoculum produced at 5 g/L glucose is more efficacious than inoculum produced at 20-40 g/L glucose.

Some glucose remained in cultures of higher initial glucose concentrations after 14 d and it was depleted completely only from the cultures with lower initial glucose concentrations (Figure 4.10). Therefore, while sufficient glucose was important to high spore yields, it did not appear that its depletion was required prior to fungal sporulation as no correlation was observed between the two factors throughout the study. Jackson and Bothast (1990), working with a strain of *C. truncatum,* showed that C depletion was not necessary for initiation of fungal sporulation. In their study, glucose concentration in liquid cultures was monitored using a high-performance liquid chromatography system and, regardless of the initial concentration, glucose was depleted from all cultures after 7 d, including those with lower initial glucose concentrations (10-40 g/L) (high sporulation), as well as others with higher initial glucose concentrations (50-80 g/L) (no sporulation). Carbon concentrations used in the current study were within the lower range tested by Jackson and Bothast (1990), but the depletion rate was lower. This may have been due to the lower incubation temperature (16°C) compared to that (28°C) used by Jackson and Bothast, which possibly affects the rate of fungal growth and nutrient exhaustion.

Unlike the previous glucose concentration experiment in this study, specific spore yields of cultures were significantly different, which may be attributed to the interaction with aeration and increased sporulation at higher aeration levels (a shaker speed of 150 RPM in regular flasks was used for the previous glucose experiment, compared to 200 RPM in baffled flasks for high aeration in this experiment). Specific spore yields of cultures with 20-40 g/L at high aeration were significantly higher than those of other treatments in this study, while specific biomass yields depended on whether the calculation was based on the initial glucose level or the amount of glucose used during fermentation. When looking at the results of the glucose concentration experiment and this glucose and aeration interaction experiment, it appears that unless a sufficient level

of aeration can be provided, an increased spore yield based on glucose concentration is merely an additive effect.

There was a weak correlation between sporulation and biomass, which generally indicates that higher amounts of biomass produced are favourable to production of higher numbers of spores. This may present a challenge, as large quantities of biomass can be a practical issue for large-scale production, including increased difficulties in spore harvest and added costs for waste disposal. Unless the mycelium can be formulated, stabilized, and used effectively as demonstrated for other biocontrol agents (Amsellem et al. 1999), this challenge remains.

Jackson and Bothast (1990) also found that increased C concentration was correlated with increased hyphal melanization or formation of compact hyphal masses similar to microsclerotia and reduced conidial formation of *C. truncatum*. In contrast, they did not observe these phenomena with *C. gloeosporioides* f. sp. *aeschynomene*, which continued to increase sporulation without melanization as C concentration was increased. In the current study, observations seemed to indicate that melanization of cultures was associated with sporulation.

Based on these results, it appears that under conditions of high aeration it would be appropriate to use a medium with a glucose concentration of 10-20 g/L, in order to avoid sacrificing specific spore yield (as does 5 g/L medium) and efficacy (as does 40 g/L medium).

5.12. Scale-up of production using 20-litre fermentors

Fungal biomass was produced under all conditions. While sporulation was low compared to preceding experiments using optimal conditions in shake-flasks, the spore yield was comparable to experiments using a similar glucose level (5 g/L) previously.

As in previous shake-culture experiments, glucose exhaustion did not appear to be the condition for fungal sporulation, which began prior to glucose depletion. It is possible that a higher glucose concentration in the medium may be beneficial for scale-up production, because glucose was exhausted quickly in the fermentation process and biomass did not increase further after glucose exhaustion. Due to poorer efficacy of the fungus in previous experiments with higher glucose concentrations, a low initial glucose

concentration (5.5 g/L) was selected for the fermentation scale-up, however it would be prudent to re-evaluate the situation using fermentors in which high aeration is achievable.

Sporulation was highest in the 30% dO fermentor, and this difference was most pronounced between 4-10 d, when the different target dO levels were achieved in each of the fermentors. This indicated that dO may be an important factor for sporulation, which corresponds to results of aeration studies in shake-flask cultures. During fermentation of *C. truncatum* using two 10-L bench-top fermentors, Slininger et al. (1993) found that different stages of the fungal life cycle (germination, mycelial growth, and sporulation) required different dissolved O_2 tensions (DOT) levels for optimal yield and/or process rates. Spores in cultures deprived of O_2 failed to germinate, while low levels (10-20% DOT) were sufficient to support germination after 6 h with a 1 h lag time, and high levels (30-80% DOT) supported similar germination after 6 h without a lag. A DOT ≥15% supported maximum specific growth rate. Sporulation was tested independent of the preceding growth stages, and they found that spore accumulation increased as DOT was increased to 55% but decreased at 75% due to possible O_2 toxicity. A DOT ≥55% supported high growth rates of mycelium but reduced sporulation. This may explain why sporulation of *C. truncatum* in the present study was actually poorer at the highest dO (60%) concentration, with the medium dO concentration (30%) producing the highest number of spores.

Culture viscosity increased after 10 d, and proper stirring could no longer be achieved at the target dO levels. The thickening of cultures was accompanied by polysaccharide production and formation of dark-coloured sclerotia in all fermentors. Cultures of *C. coccodes* became viscous after 5 d of fermentation due to the limitation of N that caused extra C sources to be converted to spore matrix (Yu et al. 1998). In this study, production of viscous polysaccharides may have been caused by similar effects, and glucose depletion may have been a result of C utilization for production of biomass, spores, as well as polysaccharides.

Further studies are required in order to replicate and repeat these results, and to induce greater sporulation of *C. truncatum* from the biomass in fermentors. This was not possible for this study due to time, material, and facility constraints at the

collaborating company. However, these preliminary results are positive, showing that growth and sporulation is possible at this scale of fermentation.

6. GENERAL DISCUSSION

One of the major constraints in early development of *C. truncatum* for biocontrol of scentless chamomile was the low spore yields using agar media for inoculum production. The potential of submerged fermentation was investigated in this study in order to resolve this problem, as the method is often suitable and/or preferred for large-scale production.

Preliminary experiments helped set the stage for the rest of the study by determining some basic parameters required to initiate culturing for investigation of various conditions under submerged fermentation. This included selection of a medium and determination of an appropriate spore concentration for initial inoculation of the liquid medium. The defined basal salts medium (DBSM) was selected for submerged fermentation of *C. truncatum* for several reasons. First of all, fungal sporulation, which was the primary consideration in design of a mass production process, was significantly higher in DBSM amended with C and N sources than in the V8 medium. DBSM was also considered to be appropriate for preliminary investigation of fermentation scale-up because ingredients in defined media are generally relatively consistent in composition and quality. Therefore individual parameters can be more easily manipulated to optimize nutritional requirements of the fungus, and growth and sporulation differences could be attributed to varying key parameters with greater confidence. However, defined media are uncommon in large-scale fermentation because they are generally quite expensive due to the use of multiple high-grade ingredients (Dahod 1999; Davis and Blevins 1979), and therefore complex substrates with similar characteristics to the defined elements may need to be considered again when more information is known about the fungus' specific nutritional requirements and an increase in production scale is desired. Although *C. truncatum* was capable of growing and sporulating in V8 media in this study, it is not expected that this will be a suitable complex substitute for large scale fermentation, as it is also expensive and is not available as a bulk commodity (Yu et al. 1997). In addition, this medium may cause difficulties in product recovery and effluent

treatment (Stanbury et al. 1995a). The optimal initial inoculum concentration for *C. truncatum* production was determined to be between $1x10^3$ and $1x10^4$ sp/ml, which is comparable to studies with a different strain of *C. truncatum* in which cultures were between $1x10^4$ and $1x10^5$ sp/ml at the beginning of fermentation (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002; Montazeri et al. 2003).

The next step in optimization of the liquid medium was to study nutrition in terms of C and N sources, followed by determination of the effect of concentration of the selected C source as well as its relative proportion to the selected N source in the medium. Both sporulation and fungal inoculum efficacy can be affected by the C and/or N sources selected for the fermentation medium (Jackson and Bothast 1990; Yu et al. 1997; Zhang et al. 2001). Similar results were found in this study, with the exception that there were not enough treatments with sufficient spore production to elucidate a difference in efficacy based on the N source. Based on overall performance, glucose and casamino acids were selected as the preferred C and N sources, respectively, for this study. Glucose has been found to be capable of supporting sporulation of *C. truncatum* in liquid cultures in other studies (Jackson and Bothast; Montazeri and Greaves). It also had the added benefit of being suitable for a method of monitoring C exhaustion used during this study.

It is likely that C requirements and optimal glucose concentrations vary between fungal species or even strains. Although higher glucose supply generally increased the spore yield, its potential negative impact on bioherbicidal efficacy of the inoculum should not be overlooked. Although it is possible that a loss in efficacy due to fermentation nutrition may be reversed through formulation during down stream processing (Bothast et al. 1993; Schisler et al. 1995), the effectiveness of this strategy on this biocontrol agent has not yet been validated. In contrast to the studies by Bothast *et al.,* (1993) and Schisler *et al.,* (1995), as well as studies by Jackson and Bothast (1990) and Montazeri and Greaves (2002), C:N ratio in this study did not have a consistent impact on sporulation of this fungus, with only one of the trials showing greater sporulation for C:N ratios between 20:1 and 25:1. While further investigation may be required to determine the effect of C:N ratio on sporulation, it is evident, based on

repeated trials, that spores produced between C:N ratios of 20:1 and 25:1 were significantly more efficacious against scentless chamomile than the inoculum produced at higher C:N ratios. This provides further reasoning for the use of this range of C:N ratio for *C. truncatum* fermentation.

Physical parameters also had an effect on sporulation, but in general the effect on efficacy of fungal inoculum was less pronounced. This strain of *C. truncatum* did not require light for growth or sporulation and did not appear to be inhibited by light exposure. The insensitivity to light may be an advantage for mass production because provision of supplementary lighting can be a challenge in large-scale production. This trait is consistent with that of other *Colletotrichum* species whose spores could also be produced in the dark (Montazeri and Greaves 2002; Montazeri et al. 2003). In the current study, cultures were incubated at relatively low temperatures because preliminary experiments had indicated that the fungus would not sporulate at 28ºC. Results of this study indicated that use of a variable temperature regime with a higher initial temperature (20ºC) that expedited development of biomass, followed by a lower temperature (12-16ºC) that was conducive to sporulation and efficacy may be more efficient for inoculum production of this fungus. Cultures are likely to be more successful at higher aeration, as indicated by the trend of greater sporulation with the use of baffled flasks on shakers with increased speed to 200 RPM (simulating higher aeration). Similar shaking speeds have been used for sporulation of other *C. truncatum* strains in submerged cultures (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson and Slininger 1993; Montazeri and Greaves 2002), indicating that a high level of aeration is beneficial for sporulation of this fungal species.

The benefit of higher glucose supply in the medium to fungal sporulation was enhanced with increased aeration. However, despite the stimulation to sporulation, higher glucose concentrations may reduce efficacy of the inoculum on scentless chamomile, indicating that conditions conducive to sporulation may have a negative impact on the fungal characteristics that are related to disease causing processes. This phenomenon is noteworthy because efficacy is imperative for success of the bioherbicide. While it is plausible to suggest a connection with a decrease in protein content caused by excess C conversion to lipid leading to lower conidial germination

(Jackson et al. 1996) or other changes in fungal conidia or extracellular contents (Montazeri et al. 2003), the mechanism for lowered efficacy of this *C. truncatum* strain against scentless chamomile is unknown.

The maximum spore yield achieved in this study was 3.86 x 10^7 sp/ml. This is comparable to spore yields achieved by Jackson and Bothast (1990), who's maximum was 3.9 x 10^7 sp/ml for a different strain of *C. truncatum*. It is feasible that higher yields may be attainable for this type of organism with further optimization, as Yu et al. (1998) achieved yields of 1.3 x 10^8 sp/ml with a strain of *C. coccodes.*

This study indicated that submerged fermentation may be a feasible means for mass production of spores of this *C. truncatum* strain, but further optimization of spore yield and maintenance of weed control efficacy within the context of a practical scale-up protocol is imperative in order for this production method to be an economically viable option. One of the positive indications of this study was that sporulation was achievable in shake-flask cultures and spore yields could be improved progressively through continued manipulation of nutritional and physical conditions. In addition, fungal growth and sporulation were shown to be possible using 20-L fermentors, although further improvements would be required to optimize the conditions and bring the spore yield to levels comparable to shake-flask cultures or possibly even higher due to the capability of aeration adjustment. It is likely that the interaction of nutrition (glucose concentration) and aeration (dissolved oxygen) will play an important role in this process, as it had a significant influence on sporulation at the shake-flask scale.

7. CONCLUSIONS

Solid agar was previously deemed to be an unsuitable medium for mass production of *C. truncatum*, and until an alternative method of culturing fungal spores of the 00-3B1 strain could be determined, some aspects of commercial investigation for biocontrol of scentless chamomile were not possible. This study has confirmed that submerged fermentation can be used to efficiently produce spores of this strain of *C. truncatum,* and indicates potential for future optimization with the goal of commercial production.

A defined basal salts medium (DBSM) amended with glucose and casamino acids was found to be an appropriate liquid medium for *C. truncatum,* with cultural initiation at a relatively low inoculum dose. Physical, environmental, and nutritional conditions were shown to have an impact on sporulation of *C. truncatum* during submerged fermentation. Glucose concentration and aeration were shown to have an impact on growth and sporulation, with the effect of glucose dramatically increased at higher aeration levels. Efficacy was also impacted by nutritional conditions, with an apparent decrease in ability of fungal infection to reduce fresh weight of scentless chamomile plants by spores produced in cultures with 40g/L glucose concentration, which was also a condition that encouraged highest sporulation. Further investigation will be required to determine optimal glucose concentration as well as the possibility to reverse the negative effect of high glucose concentration via formulation. The feasibility of scale-up of the submerged fermentation procedure was validated by showing the ability of *C. truncatum* to grow and sporulate in 20-L fermentors. Further optimization of the process is required, likely including further investigation of glucose concentration and aeration to maximize spore yield at the larger scale.

Currently, the success of submerged fermentation in shake-flasks achieved in this study has led to the development of protocols for inoculum production of *C. truncatum* for continuing laboratory, greenhouse, and field trials for biocontrol of scentless chamomile. It is also a valuable model system for development of similar fungal agents for scale-up from solid to liquid media spore production.

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APPENDIX

Table A.1. Effect of initial inoculum concentration on spore yield

Initial Inoculum Concentration (sp/ml)	Spore yield (sp/ml)			
	Trial 1		Trial 2	
1×10^3	2.32×10^6	a	1.17×10^6	a
1×10^4	1.18×10^6	a	1.36×10^6	a
1×10^5	1.19×10^6	a	6.13×10^5	b
1×10^6	3.19×10^5	a	3.16×10^5	b

[a] Means of 4 replicates from each trial. Means with the same letter(s) within a trial are not significantly different (LSD, *P*=0.05).

Table A.2. Effect of C source used in the inoculum production medium on efficacy of *C. truncatum* against scentless chamomile

Carbon source	Fresh weight (g)[a]	
Sucrose	0.68	bc
Glucose	0.56	c
Trehalose	0.68	bc
Maltose	0.90	b
Fructose	0.46	c
Control	1.58	a

[a] Means of 4 replicates from one trial. Means with the same letter(s) are not significantly different (LSD, P=0.05).

Table A.3. Effect of N source used in the inoculum production medium on efficacy of *C. truncatum* against scentless chamomile

Nitrogen source	Fresh weight (g)[a]	
Casein enzymatic hydrolysate	0.86	b
Casamino acids	1.08	ab
Control	1.30	a

[a] Means of 6 replicates from one trial. Means with the same letter(s) are not significantly different (LSD, P=0.05).

Table A.4. Effect of glucose concentration in the medium on spore yield and biomass production

Glucose concentration (g/L)	Spore yield (sp/ml)[a]		Biomass (g/L)[b]	
0	2.50×10^3	d	0.00	e
5	1.71×10^5	d	2.57	de
10	3.54×10^5	cd	3.28	de
15	3.84×10^5	bcd	3.85	cde
20	3.57×10^5	cd	6.28	bcd
25	8.56×10^5	bc	7.23	bc
30	8.78×10^5	b	8.81	b
35	1.58×10^6	a	15.80	a
40	1.82×10^6	a	19.14	a

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, P=0.05).

[b] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, P=0.05).

Table A.5. Effect of glucose concentration on specific yield

Glucose concentration (g/L)	Specific yield[a]			
	Sporulation (sp/g glucose)[b]		Biomass (g/g glucose)[c]	
5	3.43×10^7	ab	0.51	a
10	3.54×10^7	ab	0.33	ab
15	2.56×10^7	ab	0.26	b
20	1.78×10^7	bc	0.31	ab
25	3.42×10^7	ab	0.31	ab
30	2.93×10^7	ab	0.29	ab
35	4.50×10^7	a	0.45	ab
40	4.56×10^7	a	0.48	ab

[a] Means of 8 replicates from 2 trials with homogeneous variance.

[b] Sporulation means with the same letter(s) are not significantly different (LSD, P=0.05).

[c] Biomass means with the same letter(s) are not significantly different (LSD, P=0.05).

Table A.6. Effect of glucose concentration used in the inoculum production medium on fresh weight of scentless chamomile

Glucose concentration (g/L)	Fresh weight (g)[a]			
	Trial 1		Trial 2	
5	0.90	e	0.26	b
10	1.22	de	0.24	b
15	1.43	cde	0.29	b
20	1.96	bc	0.26	b
25	1.73	bcd	0.34	b
30	1.67	bcd	0.36	b
35	1.65	bcd	0.35	b
40	2.20	b	0.39	b
Control	3.61	a	1.48	a

[a] Means of 4 replicates from each trial. Means with the same letter(s) within a trial are not significantly different (LSD, P=0.05).

Table A.7. Effect of glucose concentration used in the inoculum production medium on fresh weight reduction of scentless chamomile

Glucose concentration (g/L)	Fresh weight reduction (%)[a]	
5	78.8	a
10	75.0	a
15	70.6	ab
20	64.1	ab
25	64.6	ab
30	64.7	ab
35	65.5	ab
40	56.6	b

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, P=0.05).

Table A.8. Effect of C:N ratio used in the inoculum production medium on efficacy of *C. truncatum* against scentless chamomile

C:N ratio	Fresh weight (g)[a]	
10:1	1.49	cd
15:1	1.69	bcd
20:1	1.44	cd
23:1	1.36	d
25:1	1.34	d
30:1	1.89	bc
40:1	2.08	b
Control	3.21	a

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, *P*=0.05).

Table A.9. Effect of temperature used during inoculum production on efficacy of *C. truncatum* against scentless chamomile

Temperature (ºC)		Fresh weight (g)[a]	
Week 1	Week 2		
12	12	1.35	c
12	16	1.22	c
12	20	1.43	c
16	16	1.34	c
16	12	1.32	c
16	20	1.35	c
20	20	1.97	b
20	16	1.69	bc
20	12	1.67	bc
Control		3.39	a

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, *P*=0.05).

Table A.10. Culture pH during fermentation of *C. truncatum* in liquid media with different initial pH levels

Initial pH	Culture pH during fermentation[a]		
	After autoclaving	Week 1	Week 2
4.5	4.30	4.23	4.03
5.5	5.26	5.13	5.37
6.5	5.79	5.73	6.04
7.5	6.90	6.48	7.05

Means of 8 replicates from 2 trials (pH adjusted before autoclaving).

Table A.11. Effect of shaker speed and flask type on spore yield (Trial 1)

Spore yield (sp/ml)[a]					
Shaker speed (RPM)	Flask type				Spore yield (sp/ml)[c]
	Regular		Baffled		
50	7.50×10^3	d	1.25×10^4	d	1.00×10^4 c
100	8.13×10^4	cd	2.74×10^5	bc	1.78×10^5 bc
150	2.31×10^5	bcd	4.13×10^5	ab	3.22×10^5 ab
200	3.43×10^5	b	5.94×10^5	a	4.68×10^5 ab
Spore yield (sp/ml)[b]	1.66×10^5	b	3.23×10^5	a	

[a] Means of 4 replicates from one trial. Means with the same letter(s) are not significantly different (LSD, P=0.05) for each treatment.

[b] Flask type treatment means of 16 replicates.

[c] Shaker speed treatment means of 8 replicates.

Table A.12. Effect of shaker speed and flask type on spore yield (Trial 2)

Spore yield (sp/ml)[a]					
Shaker speed (RPM)	Flask type				Spore yield (sp/ml)[c]
	Regular		Baffled		
50	1.25×10^4	d	5.00×10^3	d	8.75×10^3 c
100	2.15×10^5	cd	3.36×10^5	bcd	2.76×10^5 c
150	1.04×10^6	b	7.65×10^5	bcd	9.04×10^5 b
200	8.83×10^5	bc	2.29×10^6	a	1.59×10^6 a
Spore yield (sp/ml)[b]	5.38×10^5	a	8.48×10^5	a	

[a] Means of 4 replicates from one trial. Means with the same letter(s) are not significantly different (LSD, P=0.05).

[b] Flask type treatment means of 16 replicates.

[c] Shaker speed treatment means of 8 replicates.

Table A.13. Effect of glucose concentration and aeration on spore yield

Spore yield (sp/ml)[a]						
Glucose concentration (g/L)	Aeration				Spore yield (sp/ml)[c]	
	Low		High			
5	1.41×10^6	c	2.04×10^6	c	1.73×10^6	c
10	3.18×10^6	c	6.05×10^6	c	4.61×10^6	bc
20	4.03×10^6	c	1.47×10^7	b	9.35×10^6	b
40	3.54×10^5	c	3.86×10^7	a	1.95×10^7	a
Spore yield (sp/ml)[b]	2.24×10^6	b	1.53×10^7	a		

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, P=0.05).

[b] Aeration treatment means of 32 replicates.

[c] Glucose treatment means of 16 replicates.

Table A.14. Effect of glucose concentration and aeration on biomass production

Biomass (g/L)[a]						
Glucose concentration (g/L)	Aeration				Biomass (g/L)[c]	
	Low		High			
5	4.34	b	3.39	b	3.86	b
10	6.23	b	6.34	b	6.29	b
20	5.73	b	6.95	b	6.34	b
40	19.76	a	19.93	a	19.84	a
Biomass (g/L)[b]	9.02	a	9.15	a		

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, P=0.05).

[b] Aeration treatment means of 32 replicates.

[c] Glucose treatment means of 16 replicates.

Table A.15. Effect of glucose concentration in the DBSM and aeration level used during inoculum production on efficacy of *C. truncatum* against scentless chamomile

Fresh weight (g/L)[a]						
Glucose concentration (g/L)	Aeration				Fresh weight (g/L)[c]	
	Low		High			
5	1.83	cd	1.46	d	1.64	c
10	1.80	cd	2.16	bc	1.98	bc
20	1.95	bcd	2.27	bc	2.11	b
40	2.12	bc	2.42	b	2.27	b
Fresh weight (sp/ml)[b]	1.92	b	2.08	b	Control[d]	
					3.11	a

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, P=0.05).

[b] Aeration treatment means of 32 replicates.

[c] Glucose treatment means of 16 replicates.

Table A.16. Effect of glucose concentration and aeration on specific spore yield

Glucose concentration (g/L)	Aeration	Specific spore yield[a]			
		(sp/g initial glucose)[b]		(sp/g used glucose)[c]	
5	Low	2.83×10^8	d	2.87×10^8	d
10	Low	3.18×10^8	d	3.28×10^8	d
20	Low	2.02×10^8	de	2.35×10^8	de
40	Low	8.84×10^8	e	1.73×10^7	e
5	High	4.08×10^8	cd	4.13×10^8	cd
10	High	6.05×10^8	bc	6.10×10^8	bc
20	High	7.33×10^8	ab	7.58×10^8	b
40	High	9.64×10^8	a	1.03×10^9	a

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, P=0.05).

[b] Based on initial glucose levels.

[c] Based on amount of glucose consumed during fermentation.

Table A.17. Effect of glucose concentration and aeration on specific biomass yield

Glucose concentration (g/L)	Aeration	Specific biomass yield[a] (g/g initial glucose)[b]		(g/g used glucose)[c]	
5	Low	0.87	a	0.88	a
10	Low	0.62	b	0.67	ab
20	Low	0.29	d	0.35	c
40	Low	0.49	bc	0.89	a
5	High	0.68	ab	0.69	ab
10	High	0.63	b	0.64	abc
20	High	0.35	cd	0.36	c
40	High	0.50	bc	0.57	bc

[a] Means of 8 replicates from 2 trials with homogeneous variance. Means with the same letter(s) are not significantly different (LSD, P=0.05).

[b] Based on initial glucose levels.

[c] Based on amount of glucose consumed during fermentation.